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FAT TASTE TRANSDUCTION IN MOUSE TASTE CELLS:  
THE ROLE OF TRANSIENT RECEPTOR POTENTIAL  
CHANNEL TYPE M5

by

Pin Liu

A dissertation submitted in partial fulfillment  
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

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Logan, UT

2010

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## ABSTRACT

Fat Taste Transduction in Mouse Taste Cells:  
The Role of Transient Receptor Potential  
Channel Type M5

by

Pin Liu, Doctor of Philosophy

Utah State University, 2010

Major Professor: Dr. Timothy A. Gilbertson  
Department: Biology

A number of studies have demonstrated the ability of free fatty acids to activate taste cells and elicit behavioral responses consistent with there being a taste of fat. Here I show for the first time that long chain unsaturated free fatty acid, linoleic acid, depolarizes taste cells and elicits a robust intracellular calcium rise via the activation of transient receptor potential channel type M5. The linoleic acid-induced responses depend on G protein-phospholipase C pathway indicative of the involvement of G protein-coupled receptors in the transduction of fatty acids. Mice lacking transient receptor potential channel type M5 exhibit no preference for and show reduced sensitivity to linoleic acid. Together, these studies show that transient receptor potential channel type M5 plays an essential role in fatty acid transduction and suggest that fat may reflect a *bona fide* sixth primary taste. Studies to identify the types of taste cells that respond to

fatty acids show that both type II and type III taste cells express fatty acid-activated receptors. Fatty acids elicit robust intracellular calcium rise primarily in type II taste cells and a subset of type III taste cells. However, a significant subset of type II taste cells respond to high potassium chloride, which has been broadly used as the indicator for type III taste cells as well, suggesting the expression of voltage-gated calcium channels in these cells. This finding conflicts with previous studies that type II taste cells lack voltage-gated calcium channels. To explore if voltage-gated calcium channels are expressed in subsets of type II taste cells, transgenic mice with type II or III taste cells marked by green fluorescent proteins are used. Results show that a subset of type II taste cells exhibit voltage-gated calcium currents, verifying the expression of voltage-gated calcium channels in these cells. These results question the utility of being able to use high potassium chloride solution to identify unequivocally type III taste cells within the taste buds. A model for the transduction of fatty acids in taste cells consistent with these findings and our previous data is presented.

(155 pages)

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Pin Liu

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## LIST OF ABBREVIATIONS

AA	Arachidonic acid
ASIC	Acid-sensing ion channel
ATP	Adenosine-5'-triphosphate
BaCl <sub>2</sub>	Barium chloride
BAPTA	1,2-Bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CA	Caproic acid
Ca <sup>2+</sup>	Calcium ions
CaCl <sub>2</sub>	Calcium chloride
cAMP	Cyclic adenosine monophosphate
CD36	Cluster of differentiation 36
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
CNS	Central nervous system
CS	Conditioned stimulus
Cs <sup>+</sup>	Cesium ions
CTA	Conditioned taste aversion
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
dNTP	2-deoxynucleotide 5'-triphosphate
DRK	Delayed rectifying potassium
EC <sub>50</sub>	Half maximal effective concentration

EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
$E_{rev}$	Reversal potential
EtOH	Ethanol
FA	Fatty acid
FAT	Fatty acid transporter
FBS	Fetal bovine serum
GAD67	Glutamic acid decarboxylase <sup>67</sup>
GDP	Guanosine-5'-diphosphate
GFP	Green fluorescent protein
GHK	Goldman-Hodgkin-Katz
GLAST	Glutamate-aspartate transporter
GPCRs	G protein-coupled receptors
GTP	Guanosine-5'-triphosphate
HBSS	Hank's buffered salt solution
HCN	Hyperpolarization-activated and cyclic nucleotide-gated ion channel
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HHP	Hanks' buffered salt solution with HEPES buffer and Na pyruvate
IP <sub>3</sub>	Inositol 1,4,5-triphosphate
IP <sub>3</sub> R	Inositol 1,4,5-triphosphate receptor

I-V	Current-voltage
K <sup>+</sup>	Potassium ions
KCl	Potassium chloride
KOH	Potassium hydroxide
LA	Linoleic acid
LCFA	Long chain fatty acid
LiCl	Lithium chloride
MA	Myristic acid
MAPK	Mitogen-activated protein kinase
MCFA	Medium chain fatty acid
MgCl <sub>2</sub>	Magnesium chloride
N <sub>2</sub>	Nitrogen
Na <sup>+</sup>	Sodium ions
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NE	Norepinephrine
NTPDase	Nucleoside triphosphate diphosphohydrolase
O <sub>2</sub>	Oxygen
OA	Oleic acid
Panx1	Pannexin 1
PIP <sub>2</sub>	Phosphatidylinositol 4,5-bisphosphate



PKD	Polycystic kidney disease
PLC	Phospholipase C
PLC $\beta$ 2	Phospholipase C $\beta$ 2 isoform
PTK	Protein tyrosine kinase
qPCR	Quantitative PCR
RNA	Ribonucleic acid
ROMK	Renal outer medullary potassium
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SCFA	Short chain fatty acid
SOCE	Store operated calcium entry
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TEA	Tetraethylammonium
TM	Taste mixture
TPPO	Triphenylphosphine oxide
Tris	Tris(hydroxymethyl)aminomethane
TRP	Transient receptor potential
TRPA	Transient receptor potential ankyrin
TRPC	Transient receptor potential canonical
TRPM	Transient receptor potential melastatin
TRPM1	Transient receptor potential melastatin member 1
TRPM2	Transient receptor potential melastatin member 2

TRPM3	Transient receptor potential melastatin member 3
TRPM4	Transient receptor potential melastatin member 4
TRPM5	Transient receptor potential melastatin member 5
TRPM6	Transient receptor potential melastatin member 6
TRPM7	Transient receptor potential melastatin member 7
TRPM8	Transient receptor potential melastatin member 8
TRPML	Transient receptor potential mucolipin
TRPP	Transient receptor potential polycystin
TRPP2	Transient receptor potential polycystin member 2
TRPV	Transient receptor potential vanilloid
TRPV1	Transient receptor potential vanilloid member 1
TTX	Tetrodotoxin
US	Unconditioned stimulus
VGCC	Voltage-gated calcium channel
WT	Wild type

## CHAPTER 1

### INTRODUCTION

#### Rationale for the Proposed Research

Obesity has become one of the most serious health concerns in the world, and it is a disease that is reaching epidemic proportions especially in developed countries. In brief, obesity reflects an imbalance of energy intake and expenditure which results in excessive accumulation of body fat. Numbers posted by the National Center for Health Statistics show that more than 33.8% of Americans are obese, and another 34.2% are overweight (1). Being obese increases the incidence of various diseases, particularly diabetes, cardiovascular disease, end-stage renal disease, and certain types of cancer. While obesity has clearly been a leading cause of death worldwide, it is not surprising that there have been numerous studies during the past few decades exploring the underlying mechanisms of obesity and the factors that contribute to the development of obesity. One of the precipitating factors that has been suggested to play a role in the development of dietary-induced obesity is the increase in dietary fat intake (2-5).

Yet, is fat the enemy of our health? Clearly the overconsumption of fat, along with the lack of exercise, leads to increased body mass, which represents an unhealthy lifestyle. At the same time, however, fats are critical players for many biological processes. Fats are the primary components of the lipid bilayer of cells, which is considered to be the basic building blocks of life. Fats provide the insulation that helps maintain body temperature and protect important organs from damage. Fats also serve as the solvent for Vitamin A, D, E, and K which can only be digested, absorbed, and

transported in conjunction with fats. Clearly, fat is critical for life and health. Furthermore, our body cannot produce certain kinds of fats, termed essential fatty acids, from other compounds, indicating that they need to be consumed in the diet. Thus the ability to detect these fats in food sources is necessary for survival.

In general, fatty foods are highly palatable, providing strong motivation for mammals, including humans to search for and consume fat-containing foods. Studies have shown that most mammals, including humans, prefer foods abundant in oil and fats. So why is fat so tasty? For many years, it was considered that fat and the components contained therein were mainly perceived by their textural and smell properties through the activation of somatosensory and olfactory system, respectively. Studies have shown that increasing the texture of low-fat food increased the perceived fat content. On the contrary, animals with impaired ability to smell lost their preference for high-fat food (6). However, when the effects of texture, olfaction and postingestive effects were minimized, rats can still discriminate different oils and continued to prefer fat solutions, suggesting that fat might provide salient cues to the gustatory system as well (7).

During the last decade, there has been accumulating evidence demonstrating the ability of components in fats, specifically free fatty acids, to activate taste cells, consistent with there being a “taste of fat.” Gilbertson *et al.* provided the first evidence that free fatty acids activate taste receptor cells by inhibiting delayed rectifying potassium (DRK) channels (8-10). More recently, several additional fatty acid-responsive proteins have been identified that may play a role in initiating fatty acid transduction, including the fatty acid binding protein, CD36 (11-14) and several G protein-coupled receptors

(GPCRs) (15-20). Despite the findings of these putative fatty acid-responsive proteins, the underlying mechanisms for fat transduction have not been unequivocally elucidated. My dissertation research was therefore designed to study the fat taste transduction pathway in mammalian taste cells using mouse as the animal model.

### Peripheral Gustatory System and Taste Transduction

The gustatory system enables animals to detect and distinguish between safe and dangerous food, to select the nutritious content of food, and to prevent the ingestion of toxic substances. For most mammals, especially humans, the decision to ingest a particular food depends not only on its taste but also on its texture, appearance, odor, etc., all of which contribute to the overall enjoyment of a meal.

Surprisingly, although we can taste a variety of chemical entities, it is well accepted that they evoke only a few distinct taste sensations: salty, sweet, bitter, sour, and umami (“meaty” or “savory” in Japanese). Each of the taste sensations represents different nutritional or physiological requirements. Sweet taste signals the presence of carbohydrates. Salty taste signals the intake of minerals, primarily sodium ions and other salts, which play essential roles in maintaining the salt/water balance of the body. Umami taste detects a few L-amino acids, mainly L-glutamate, reflecting the protein content in the food. Bitter taste helps to prevent consuming toxins and poisons in the food. Sour taste detects the presence of acids which we want to avoid in order to maintain the acid-base balance of the body. Moreover, spoiled foods usually taste sour and are avoided.

Generally speaking, sweet, salty, and umami tasting foods provide a pleasant sensation, while bitter and sour foods are typically deemed unpleasant. In addition, recent studies suggest that there are numerous non-primary tastes that exist in the peripheral gustatory system, such as fatty, astringency, water, metallic, cooling, and pungent.

### **Organization of the Mammalian Gustatory System**

The anatomical substrates and units of taste detection are epithelial-derived taste cells (21-23). As the first step the body uses to detect and ingest food, it is clear that the taste cells play a critical role in the selective acceptance or rejection of food. As a result, it is not surprising that taste cells, assembled into organelles called taste buds, could detect and identify numerous different chemical stimuli that correspond to these primary and non-primary tastes.

Taste cells are clustered into different types of taste buds. The majority of taste buds on the tongue sit on raised protrusions of the tongue surface called papillae. Based on the different morphological structures, there are four types of papillae present in the mammalian tongue, three of which contain taste buds and are then involved in gustation. Fungiform papillae are mushroom shaped and present mostly on the dorsal surface at the anterior two-thirds of the tongue. Foliate papillae are ridges and grooves on lateral margins towards the posterior part of the tongue. Circumvallate papillae are arranged in a circular-shaped row and present at the back of the tongue. All three of these taste papillae mentioned above contain taste buds and are involved in gustation. Another type of

papillae, filiform papillae, do not contain taste buds. They are considered to be mechanical and not directly involved in taste sensation (24, 25) (Figure 1.1).

Mammalian taste cells are not neurons and thus do not send axonal projections to the brain. Instead, they generate action potentials and release various neurotransmitters in response to taste stimuli, and this signal is then transmitted by neurons that innervate taste buds. Each taste bud is innervated by 3-14 sensory ganglion neurons, depending on the species and oral region (26, 27). Meanwhile, each primary gustatory nerve fiber innervates multiple taste cells, within a taste bud or from different taste buds. The cell bodies of these sensory neurons are located in clusters nestled against the brain (the geniculate, petrosal, and nodose cranial ganglia). Sensory fibers from the chorda tympani (cranial nerve VII) contact fungiform papillae at the anterior tongue, and those from the greater superficial petrosal branch (cranial nerve VII) innervate taste papillae at the palate. Fibers from the lingual branch of the glossopharyngeal (cranial nerve IX) innervate foliate and circumvallate papillae, while those from the superior laryngeal branch of the vagus nerve (cranial nerve X) innervate the epiglottis and larynx. The three nerves relay taste information to the rostral and lateral regions of the solitary tract nucleus of the medulla in a topographical manner. The chorda tympani projections are rostral to glossopharyngeal fibers, which are rostral to the superior laryngeal fibers. Gustatory information is then transferred from the solitary tract nucleus to the thalamus, and then to gustatory areas of the cortex (24, 28) (Figure 1.2).

### **Taste Cells Are of Discrete Types**

Taste buds, which are distributed across different papillae of the tongue, are collections of approximately 50-150 taste cells specialized for the detection of different taste stimuli in the aqueous saliva through a small taste pore. Based on ultrastructural features, taste bud cells are classified into cell types, including basal cells, type I, II, and III taste cells, the functions of which have not been firmly established (29-39) (Figure 1.3).

Basal cells are likely to be proliferative or immature taste cells that give rise to new taste cells. They do not extend processes into the taste pore. The exact functions of basal cells remain to be elucidated.

Type I taste cells are the most abundant cells in taste buds. They ensheath surrounding cells (38) and their primary functions are to support type II and III taste cells. They express renal outer medullary potassium (ROMK) channels on the apical membrane (40), which have been proposed to contribute to the regulation of potassium ions around cells of the taste bud. When intense taste stimulation induces trains of action potentials, potassium ions may accumulate in the limited intercellular spaces in the taste buds and prevent the excitability of type II and III taste cells. Type I taste cells via ROMK channels may help clear out the potassium ions in the intercellular spaces, leaving type II and III taste cells ready for another excitation. Type I taste cells also express the glutamate-aspartate transporter (GLAST) (41), a glutamate transporter which may be involved in glutamate uptake, and nucleoside triphosphate diphosphohydrolase (NTPDase) (42), which is a plasma membrane-bound nucleotidase that hydrolyzes



extracellular ATP. Since both glutamate and ATP serve as neurotransmitters in taste buds, type I taste cells are considered to limit the diffusional spread of neurotransmitters and terminate synaptic transmission. Their wrapping around neighboring cells would further limit the diffusion of neurotransmitters mentioned above. Recent studies suggest that type I taste cells might also directly respond to salty taste (43).

Type II taste cells are regarded to be taste receptor cells, which contain receptors and signaling components for sweet, bitter, and umami tastes. These receptors are GPCRs with seven transmembrane segments. Binding of taste compounds to their corresponding receptors activate a signaling pathway including the activation of G proteins and phospholipase C (PLC), release of calcium from intracellular stores, and opening of transient receptor potential channel type M5 (TRPM5) (44-47). They also express voltage-gated sodium and potassium channels which are essential for firing action potentials. Recent studies show that they secrete ATP as the neurotransmitter through hemichannels, and pass information to Type III taste cells or directly onto the gustatory afferent nerves (48-52). Interestingly, they do not form conventional synapses onto afferent nerve fibers. Instead, ATP secreted by type II taste cells diffuses to the nearby gustatory afferent nerves that are closely apposed to these cells (53-56) and activates ATP receptors. There are studies showing that taste receptor cells, presumably type II taste cells, can respond to multiple taste stimuli (57). However, more recent studies support the idea that type II taste cells are narrowly tuned. In other words, one given type II taste cell only responds to one taste quality (58).

Type III taste cells express synaptic proteins and form synaptic junctions with nerve fibers (30, 52-55) which then transfer taste information to the central nervous system. Thus they are usually labeled presynaptic cells. They are excitable and express voltage-gated sodium and potassium channels to help fire action potentials (59-61). They also express voltage-gated calcium channels (VGCCs) which are closely related to neurotransmitter release (62). Recently, some studies show that type III taste cells may also directly participate in taste transduction. They specifically respond to sour taste stimuli and carbonated solutions and secrete serotonin as the neurotransmitter (58, 63-66). Besides the direct sensation of sour and carbonated taste stimuli, type III taste cells also receive ATP and integrate this information generated by type II taste cells. Thus type III cells are not narrowly tuned to one taste quality but instead respond broadly to all five conventional taste qualities (58).

### **Molecular Mechanisms for Taste Transduction**

How are the different taste stimuli detected? Initially, different taste compounds must diffuse through the saliva covering the taste pore to interact with the apical membrane of the taste cells. As described above, sweet, bitter, and umami compounds activate different GPCRs which are expressed in discrete subsets of type II taste cells. Type II taste cells that express the heterodimer T1R2+T1R3 respond to sweet taste (67-69); type II taste cells expressing the T2R family of GPCRs respond to bitter compounds (70); while type II taste cells that express the heterodimeric GPCRs, T1R1+T1R3, respond to umami compounds (71, 72). When these GPCRs are activated by the binding

of taste compounds, they activate G proteins and cause the dissociation of the  $G_\alpha$  subunits from the  $G_{\beta\gamma}$  subunits (73).  $G_{\beta\gamma}$  subunits then interact with PLC $\beta$ 2, a specific isoform of PLC, which in turn catalyzes the reaction to cleave phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP<sub>3</sub>) (74-76). IP<sub>3</sub> binds to IP<sub>3</sub> receptors (IP<sub>3</sub>R) on the endoplasmic reticulum and releases calcium into the cytosol of type II taste cells (46, 47). The elevated intracellular calcium or the depletion of intracellular calcium stores activates monovalent cation channel TRPM5 whose activation further depolarizes the cell via sodium influx (77, 78). This depolarization, together with the increased intracellular calcium concentration, opens gap junction hemichannels, likely composed of pannexin 1 (Panx1) (49, 50, 79), causing the release of ATP as neurotransmitters through the hemichannel pores into the extracellular space (49, 50, 80, 81) (Figure 1.4A).

Different from the transduction of sweet, bitter, and umami tastes, sour taste compounds (acids) activate type III taste cells (58). Many possible sour taste receptors have been identified. The acid-sensing ion channels (ASICs) are well established as comprising cation channels that are activated by extracellular protons (82). Hyperpolarization-activated and cyclic nucleotide-gated ion channels (HCNs) may activate a G protein-activated pathway that reduces the intracellular calcium rise in response to the sour stimulus (47). Recent additions to the gallery of sour taste receptors are the nonselective cation channels formed by PKD2L1 and PKD1L3 (83-85), two members of the polycystic kidney disease (PKD or TRPP) family of transient receptor potential (TRP) channels, and certain potassium channels that respond to cytoplasmic

acidification (86, 87). Previous studies show that the proximate stimulus for sour taste is a drop in cytoplasmic pH (63), suggesting that certain potassium channels are more likely to be the candidate receptors. On the contrary, PKD2L1 and PKD1L3 are more sensitive to extracellular pH rather than intracellular acidification. Consistent with this, mice lacking PKD1L3 retain normal taste responsiveness to sour taste in both behavioral and electrophysiological tests (88), which questions the involvement of these TRP channels in sour taste. Thus the mechanism for sour taste likely involves the acidification of the cytosol, blocking of certain proton-sensitive potassium channels which in turn depolarizes the membrane, opening of VGCC channels that allows the influx of calcium ions, and neurotransmitter release triggered by elevated cytoplasmic calcium level (Figure 1.4B).

Taste cells detect salty taste mainly through the amiloride-sensitive epithelial sodium channel, ENaC, which is located on the apical membrane (89-91). The influx of sodium ions through ENaC channels depolarizes the membrane and triggers downstream signaling pathways (Figure 1.4C). In addition to the well-defined amiloride-sensitive pathway in which ENaC plays an essential role, there seems to be an amiloride-insensitive pathway which also contributes to salty taste. Transient receptor potential vanilloid member 1 (TRPV1) has been suggested as the salt receptor in this amiloride-insensitive pathway (47). The cell type that underlies salty taste is not clear yet. But electrophysiological experiments suggest that type I cells are involved in the detection of salty compounds (43).

### **Cell-to-Cell Information Processing in Taste Buds**

There are several neurotransmitters involved in the taste signaling. Type II taste cells secrete ATP through hemichannels, while type III taste cells release serotonin and norepinephrine (NE) through conventional vesicular exocytosis (92, 93).

When type II taste cells are activated by sweet, bitter, and umami taste stimuli, they secrete ATP onto afferent nerve fibers and/or adjacent type III taste cells which in turn release serotonin and/or NE. ATP can also stimulate type II taste cells that release it through an autocrine mechanism and increase its own secretion which represents a positive feedback (94).

Type III taste cells, on the other hand, secrete serotonin and/or NE when they receive ATP from type II taste cells, or are directly activated by sour taste or carbonated solutions. The released serotonin and NE stimulate their receptors on afferent nerves which then transfer this information to the central nervous system. Recent studies show that serotonin can also exert a negative feedback onto type II taste cells and inhibit the activation of these cells (94). The combined action of serotonergic paracrine and purinergic autocrine mechanisms may underlie the modulation of gustatory signaling in the taste system (Figure 1.5). While the communication between different types of taste cells within the taste bud sounds attractive, a lot of details remain unclear and to date it cannot completely explain peripheral gustatory function.

## TRPM5 Channel Plays an Essential Role in Taste Transduction

### Introduction to TRP Channels

TRP channels form a large gene family of ion channels that have distinct activation mechanisms and biophysical properties. The first TRP channel was discovered in studies that examined *Drosophila* phototransduction (95). The photoreceptor cells of *Drosophila* exhibit sustained receptor potentials to continuous light exposure, which is due to the influx of calcium from the extracellular space. In 1969, literature reported that one group of mutant flies exhibited a transient voltage response to continuous light, and named it *trp* for transient receptor potential (95). In 1989, Montell *et al.* first cloned the *trp* gene (96), and subsequent studies showed that *trp* gene encoded a calcium-permeable cation channel (97). Since then, a number of channels, which have similar sequence and structure to the *Drosophila* TRP, have been found and cloned from worms, flies, and mammals, and form a huge TRP superfamily.

Unlike most ion channels that are identified by their ion selectivity and mechanism of activation, TRP channels are identified only by their homology. One reason is that their functions are very disparate and often unknown or unclear. Some TRP channels are activated by ligands, while others are activated by physical stimuli and involved in thermosensation, mechanosensation, smell and taste. Studies shows that yeast use a TRP channel to respond to hypertonicity (98, 99). Male mice use a TRP channel to tell males from females (100). Humans and other mammals use TRP channels to sense sweet, bitter, and umami tastes (75), and to distinguish heat and cold.

All of the TRP channels have four putative six-transmembrane segments and they assemble into tetramers to form cation-permeable pores. TRP channels are ubiquitously expressed, and most cells have more than one type of TRP channel.

According to the sequence homology, mammalian TRP channels fall into six subfamilies: TRPC, TRPV, TRPM, TRPML, TRPP, and TRPA (Figure 1.6) (101). Due to the low similarity of the transmembrane segments, the sequence identity across the entire superfamily is only 20%. Within each subfamily, the sequence identity is much higher.

More recently, various studies show that TRP channels play a critical role in taste perception. PKD2L1 (TRPP2) and PKD1L3 are expressed in type III taste cells and are involved in sour taste transduction (82-84). TRPM5, which is expressed in type II taste cells, is shown to be essential for sweet, bitter, and umami taste perception (75).

### **TRPM5 Channels**

The TRPM (melastatin) subfamily is named according to the founding member, melastatin (TRPM1). Until now, eight mammalian members have been found in the TRPM subfamily. TRPM subfamily contain TRP domain in the C-terminus, which is considerably longer than the corresponding region of other TRPs. Some TRPM members contain enzyme domains in the C-terminus, and are called “chanzymes”. TRPM subfamily includes TRP channels that have disparate functions. TRPM2 is a calcium-permeable channel and is important in sensing oxidative stress and related to the metabolism of ADP-ribose and nicotinamide adenine dinucleotide (NAD) (102-104). TRPM3 is also a calcium-permeable, nonselective cation channel whose activity is

increased by hypotonicity (105). TRPM8 is a nonselective, outward rectifying channel and can be activated by cold (8~28°C) and enhanced by cooling compounds like menthol (106, 107). TRPM6 is a channel and may be involved in the magnesium uptake in kidney and intestine (108). TRPM7 is permeant to both calcium and magnesium, and is sensitive to physiological magnesium-ATP levels, which suggests a role in metabolic sensing or magnesium homeostasis (108-111). Unlike all the other TRP channels, TRPM5 and TRPM4 are the only TRP channels that are permeable to monovalent cations but not calcium ( $P_{Ca}/P_{Na} < 0.05:1$ ) (112, 113). In 2002, Perez *et al.* reported that TRPM5 is highly expressed in taste tissues (77), and implicated that TRPM5 may play an essential role in the transduction of sweet, bitter, and umami tastes (75, 114, 115). Since then, more studies suggest that it is especially important for the eventual release of neurotransmitters from the taste cells and activation of gustatory afferents (116). Interestingly, recent studies show that TRPM5 is sensitive to temperature, pointing to its role in “thermal taste,” i.e. enhanced sweet perception with increasing temperature (117).

As stated in the “Molecular Mechanisms for Taste Transduction” section of this chapter, stimuli for sweet, bitter, and umami taste bind to GPCRs on the apical surface of type II taste cells, activate PLC $\beta$ 2, leading to hydrolysis of PIP<sub>2</sub> to DAG and IP<sub>3</sub>, release of intracellular calcium, and activation of the monovalent cation channel TRPM5 (46, 47, 73-78). Behavioral experiments showed that mice with a targeted deletion of the PLC $\beta$ 2 gene exhibited diminished sensitivity to sweet, bitter, and umami stimuli (75, 76), which directly supports the importance of phosphoinositide signaling in taste transduction. Then similar experiments showed that mice with a targeted deletion of TRPM5 could not



detect physiologically relevant concentrations of sweet or bitter taste stimuli (75, 115), and supported the idea that TRPM5 is critical for this signaling pathway. So, it is hypothesized that taste transduction involves the PLC signaling cascade which leads to the activation of TRPM5 channels, a signaling pathway very similar to phototransduction in fly eyes.

How is TRPM5 regulated? Until now, the idea of how TRPM5 is regulated by PLC signaling is still contradictory. Some studies showed that TRPM5 channels can be directly activated by intracellular calcium (78, 113, 118) or the depletion of calcium from intracellular stores (77). Other studies showed that TRPM5 is not activated by calcium, IP<sub>3</sub>, or depletion of intracellular calcium stores (75). Since some of the studies were done in cell lines, it remains possible that the heterologously expressed channels do not fully recapitulate the properties of native channels (119), making the mechanisms even more uncertain.

What is the exact role of TRPM5 in the taste signaling pathway? Mechanistically, the role of TRPM5 in taste transduction is poorly understood. As discussed above, TRPM5 is activated by G protein-PLC signaling, but the molecular mechanisms following TRPM5 activation are still unknown. Recent studies suggested that the opening of TRPM5 channels generate receptor potentials (77, 78). And if the receptor potentials are sufficiently large, they will evoke action potentials in type II taste cells. Furthermore, it is still unknown whether TRPM5 is associated with GPCR signaling elements as one functional unit due to sub-cellular compartmentalization, or it is independently localized in specialized sub-cellular compartments.

## The Perception of Fat in the Gustatory System

### **DRK Channels Work as Fatty Acid Receptors**

As mentioned before, for a long time, it was widely accepted that fat is tasteless and its most salient cue is its texture (120, 121) which is usually described as “oiliness,” “creaminess,” “mouthfeel,” or “slipperiness.” However, based on the facts that taste system can detect compounds necessary for survival, people began to hypothesize that the gustatory system “should” be able to detect fat, specifically the essential fatty acids in the diet.

Around twenty years ago, researchers found that free fatty acids can work as specific extracellular messengers or signals and activate a variety of systems (e.g. smooth and skeletal muscle, cardiac cells) through the inhibition of DRK channels (122-124). Later studies showed that free fatty acids exist in significant amount in fat-containing foods. Moreover, when fat-containing foods are consumed, a large amount of free fatty acids can be generated and transported in the oral cavity through the action of lingual lipase and the von Ebner’s gland proteins. Lingual lipase, the enzyme responsible for efficient free fatty acid release from dietary triglycerides, is capable of hydrolyzing ~70  $\mu\text{mol}$  of triglycerides per minute in rats (125). Von Ebner’s gland proteins, which are structurally similar to lipocalins, a family of proteins that play critical roles in the transport of lipophilic molecules (126, 127), bind no other taste molecules other than free fatty acids (128-130).

Based on these observations, we began to use free fatty acids as the prototypical taste stimulus and examined if they can activate the taste cells in a manner similar to how they act in other systems. Interestingly, this has been proven to be the case. Gilbertson *et al.* were the first to show that free fatty acids could activate taste cells through the inhibition of DRK channels (8), which would in turn depolarize taste cells. The effective concentration of fatty acids fell well in the range present in fat-containing foods or that can be produced during fat consumption (125). The depolarization induced by the action of fatty acids has two roles. The first role is to modulate the responses of taste cells to other taste qualities. Since DRK channels help repolarize the membrane following the activation, the inhibition of these channels by fatty acids enhances and prolongs the depolarization, which has been verified in electrophysiological experiments (8). Consistent with the findings at the cellular level, behavioral tests showed that *cis*-polyunsaturated free fatty acids (linoleic acids) were able to alter the preference for a subthreshold concentration of saccharin, a sweet compound. Neither saccharin nor linoleic acid at the concentration tested was preferred when given alone. However, the combination of these two stimuli was significantly preferred by the animals (10). The second role of the fatty acid-induced depolarization is to act as the signal of fat taste when other taste stimuli are absent, in other words, when fatty acids act as the primary taste quality. However, one problem with this model is that fatty acids act as open channel blockers (122, 124, 131, 132). As a result, for fatty acids to activate taste cells as a primary taste quality, at least a proportion of DRK channels need to be open at resting membrane potential. However, data showed that only a small proportion (approximately

5%) of DRK channels would be open at the resting membrane potential of taste cells (around -35 to -55 mV) (133). This apparent contradiction led us to examine if there are additional receptors and signaling pathways upstream of the DRK channels. This became the major aim of this dissertation research.

### **Other Fatty Acid Receptors**

Recently, several additional fatty acid-responsive proteins have been identified that may be involved in the initiation of fatty acid transduction, including the fatty acid binding protein, CD36 (11-14), and several GPCRs whose expression in the taste system has been verified (15-20).

#### CD36

CD36 (cluster of differentiation 36) is considered to be a transmembrane protein that binds lipids, especially long chain fatty acids (LCFAs), with an affinity in the nanomolar range (11, 134). In 1997, Fukuwatari *et al.* identified that the membrane fatty acid transporter (FAT), which is 85% similar to human CD36 glycoprotein, is expressed in the circumvallate papillae of the tongue in rat (12). The CD36 amino acid sequence predicts a ditopic glycoprotein with a large extracellular hydrophobic pocket (135, 136) between two short cytoplasmic tails. Since the C-terminal cytoplasmic tail has been shown to be associated with Src kinases (137), it is very likely that CD36 is involved in cell signaling. In 2005, Laugerette *et al.* provided more evidence suggesting an important role of CD36 in gustatory fat perception (13). Using qPCR, they showed that CD36 was strictly restricted to the lingual gustatory papillae, and was highly expressed in

circumvallate papillae compared to foliate and fungiform papillae. Using both wild type and CD36 knockout mice in the behavioral experiments, they showed that wild type mice exhibited a strong preference for a linoleic acid-enriched solution. On the other hand, loss of CD36 eliminated the preference for linoleic acid over the control solution (13). Recently, using isolated CD36-positive cells from mouse circumvallate papillae, El-Yassimi *et al.* have shown that linoleic acid, an unsaturated LCFA, binds to mouse CD36-positive gustatory cells, triggers Src-PTKs phosphorylation, raises the intracellular calcium concentration, and evokes the release of neurotransmitters, which in turn transmit the information to the afferent nerve fibers and eventually to the central nervous system (138).

### GPCRs

GPCRs are key regulators for a number of physiological functions. The human genome encodes for 865 GPCRs (139, 140). Recently, several of these GPCRs were identified as receptors for free fatty acids. One of the recently characterized GPCR families is GPR40-43, including GPR40, GPR41, and GPR43 (141). These three members are closely related to each other and share 30~40% sequence identity. Other GPCRs, such as GPR120 (142) and GPR84 (143), are also found to be activated by fatty acids. I will discuss them separately below.

In 2003, three groups (144-146) identified GPR40 or free fatty acid receptor (FFA1R) independently. Using intracellular calcium signaling assays, GPR40, which is predominantly expressed in pancreatic  $\beta$  cells (147) and the brain (144), is found to be a receptor for medium (C6-C12) and long chain fatty acids (C14-C24). Both saturated

(C12-C16) and unsaturated fatty acids (C18-C20) are capable of activating GPR40 with the carboxyl group of the fatty acids (16, 144-146). Several groups have addressed the mechanism of GPR40 (148-151) activation by fatty acids. They suggested that GPR40 is coupled to G-protein subunit  $G_{\alpha q/11}$ . When it is activated by fatty acids, GPR40 increases intracellular calcium levels and activates mitogen-activated protein kinase (MAPK) and PLC (144).

GPR41 (FFA3R) and GPR43 (FFA2R) are activated by short chain fatty acids (SCFAs;  $C \leq 6$ ) (152-154), but their specificity for SCFA, tissue locations, and physiological functions are different. The optimal carbon length is 3~5 for GPR41 and 2~3 for GPR43. GPR41 is broadly expressed, highest in brain, lung, and adipose tissue (155-157). Some studies showed that GPR41 has a function related to leptin secretion (157), but some other studies showed contradictory results. GPR43 is found in leukocyte and adipose tissue, suggesting a potential role for GPR43 in immune cell function and haematopoiesis (152, 158). The pathways activated by GPR41 or GPR43 include  $IP_3$ , release of intracellular calcium, and inhibition of cAMP accumulation (153). GPR42, another member of this family, is probably a result of gene duplication of GPR41 and is not activated by SCFAs (141).

GPR120 has been recently identified as a receptor for fatty acids. LCFAs including saturated fatty acids (C14-C18) and unsaturated fatty acids (C16-C22) were found to be ligands of GPR120 (142). It is abundantly expressed in mouse and human intestinal tract and intestinal endocrine cell line STC-1 (159). Since STC-1 cells express

similar arrays of fatty acid- and tastant-activated GPCRs as taste cells, it is plausible that GPR120 may play some role in the fat perception in the gustatory system as well.

In 2006, another GPCR, GPR84 was shown to be activated by medium chain fatty acids (MCFAs) (C9-C14). When activated by MCFAs, GPR84 induces cAMP increase and calcium mobilization in the cell (143). GPR84 is expressed in granulocytes, neutrophils, eosinophils, and peripheral blood monocytes, suggesting that it may function in fatty acid-mediated immune regulation.

#### **Working Model: Hypothesized Fat Taste Transduction Pathway**

Since fat can apparently activate the gustatory system, the question becomes what is the underlying mechanism for fat taste transduction? As stated above, a number of proteins may function as fatty acid receptors or transporters in the peripheral gustatory system. These include the fatty acid binding protein, CD36, fatty acid-sensitive DRK channels and fatty acid-activated GPCRs. Based on preliminary data generated in our laboratory using cell-based assays and pharmacological tools, I have hypothesized a model shown in Figure 1.7 that can link all these proteins together. Fatty acids, either transported by CD36 to the GPCRs or directly bound to GPCRs activate G proteins that stimulate PLC $\beta$ 2, which in turn catalyzes the reaction to generate two second messengers, DAG and IP<sub>3</sub>. IP<sub>3</sub> binds to IP<sub>3</sub>R on the endoplasmic reticulum and releases intracellular calcium. The direct binding of calcium ions or the depletion of intracellular calcium stores activates the monovalent cation channel TRPM5 whose activation further depolarizes the cell via sodium influx. This depolarization opens voltage-dependent, fatty

acid-sensitive and fatty acid-insensitive DRK channels. Inhibition of fatty acid-sensitive DRK channels by fatty acids further reinforces and prolongs the depolarization. This depolarization opens VGCCs leading to a calcium rise in the cytosol. The combined action of depolarization and elevated cytoplasmic calcium level eventually causes the release of neurotransmitters.

### Dissertation Outline

In recent years, significant advances have been made in our understanding of the cellular and molecular mechanisms for the transduction of the complex taste stimuli (e.g. sweet, bitter, and umami). However, the mechanisms underlying fat taste perception, which is tightly linked to obesity, remains elusive. Thus, it is important to elucidate the mechanisms that our body uses to recognize dietary fat.

Until now, our work and the work of others have implicated a variety of important proteins in the recognition of fatty acids, the prototypical fat stimulus. These include CD36, the fatty acid transporter, fatty acid-sensitive DRK channels, fatty acid-activated GPCRs (GPR-40, -41, -43, -84, -120), and TRPM5 cation channels. The goal of this dissertation research then is to explore the fatty acid signaling pathway in mammalian taste cells. Moreover, my preliminary data implicates TRPM5 channels are essential in this signaling pathway. Thus the focus of this dissertation project is on the role of TRPM5 in the fatty acid signaling pathway in mammalian taste cells. The hypothesis I will test is that fatty acid binding to fatty acid-activated GPCRs leads to the activation of



TRPM5, which is necessary to produce the receptor potential (depolarization) required to active taste cells during fat stimulation.

In chapter 2, my research is focused on the mechanisms that underlie the taste of fat, i.e. the transduction pathway of fatty acid in mouse taste cells. First, I used molecular approaches including reverse transcriptase PCR (RT-PCR) and quantitative real time PCR (qPCR) and identified rich arrays of fatty acid-activated proteins including CD36, DRK channels, and several fatty-acid responsive GPCRs (GPR-40, -41, -43, -84, -120) in mouse taste cells. Then I used cell-based assays including both patch clamp recording and functional calcium imaging with pharmacological approaches to explore the functional aspects in this pathway. Furthermore, a transgenic mouse model that lacks TRPM5 (115) was used in both electrophysiological and behavioral tests to determine the role of TRPM5 channels in this process.

In chapter 3, my research is focused on exploring which type of taste cells responds to fatty acids. Recently, several solutions have been widely used as “indicator” solutions to determine taste cell types. Responses to a taste mixture which contains sweet, bitter, and umami compounds were used to identify type II taste cells, and responses to high KCl solutions were used to determine type III taste cells, those expressing VGCCs (58, 93, 94). In this study, I used the “indicator” solutions and followed the procedure published to determine the subtypes of taste cells that respond to fatty acids. Results were verified using transgenic mice expressing enhanced green fluorescent protein (GFP) under control of the PLC $\beta$ 2 (GFP-PLC $\beta$ 2) (160) or GAD67 (GFP-GAD67) (58, 161) promoter in which type II or type III taste cells were marked by GFP, respectively.

Surprisingly, here I found that a significant subset of type II taste cells also respond to high KCl, suggesting the expression of VGCCs in these cells, which conflicts with previous studies that type II taste cells lack VGCCs. This unexpected observation was further studied using GFP-PLC $\beta$ 2 mice and to date my data strongly suggest that VGCCs are expressed in a subset of type II taste cells. Fatty acids used in the study vary in their chain length and degree of unsaturation, which may provide a broader view of the “taste of fat.”

In Chapter 4, I summarize my research and discuss questions reflected by this study. Future directions are also provided in this chapter.

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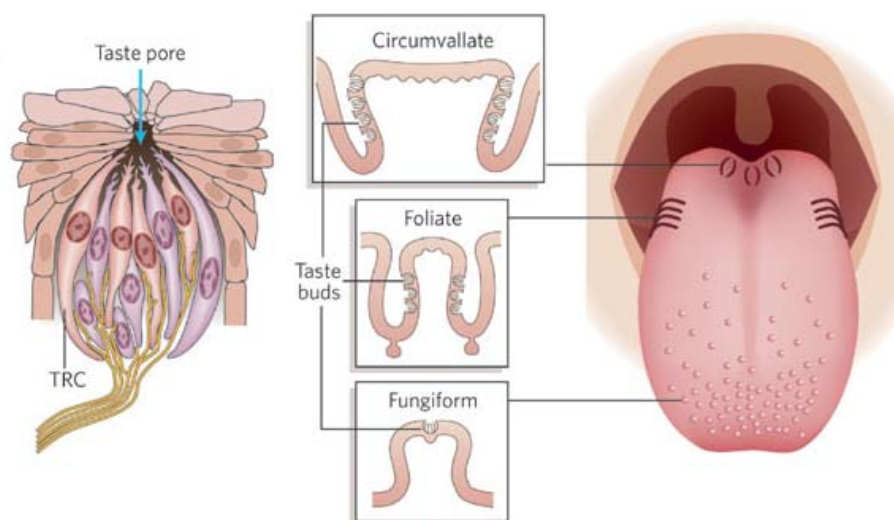


Figure 1.1 Taste buds and papillae. Taste buds (left) are composed of 50-150 taste cells distributed across different papillae. Taste cells project microvilli to the apical surface, where they form the “taste pore”, the site of interaction with taste stimuli. There are three types of taste papillae/buds on the tongue that are involved in taste sensation. Fungiform papillae present at the anterior two-thirds of the tongue. Foliate papillae are ridges and grooves on the lateral margins towards the posterior part of the tongue. Circumvallate papillae are arranged in a circular-shaped row and present at the back of the tongue. (25)

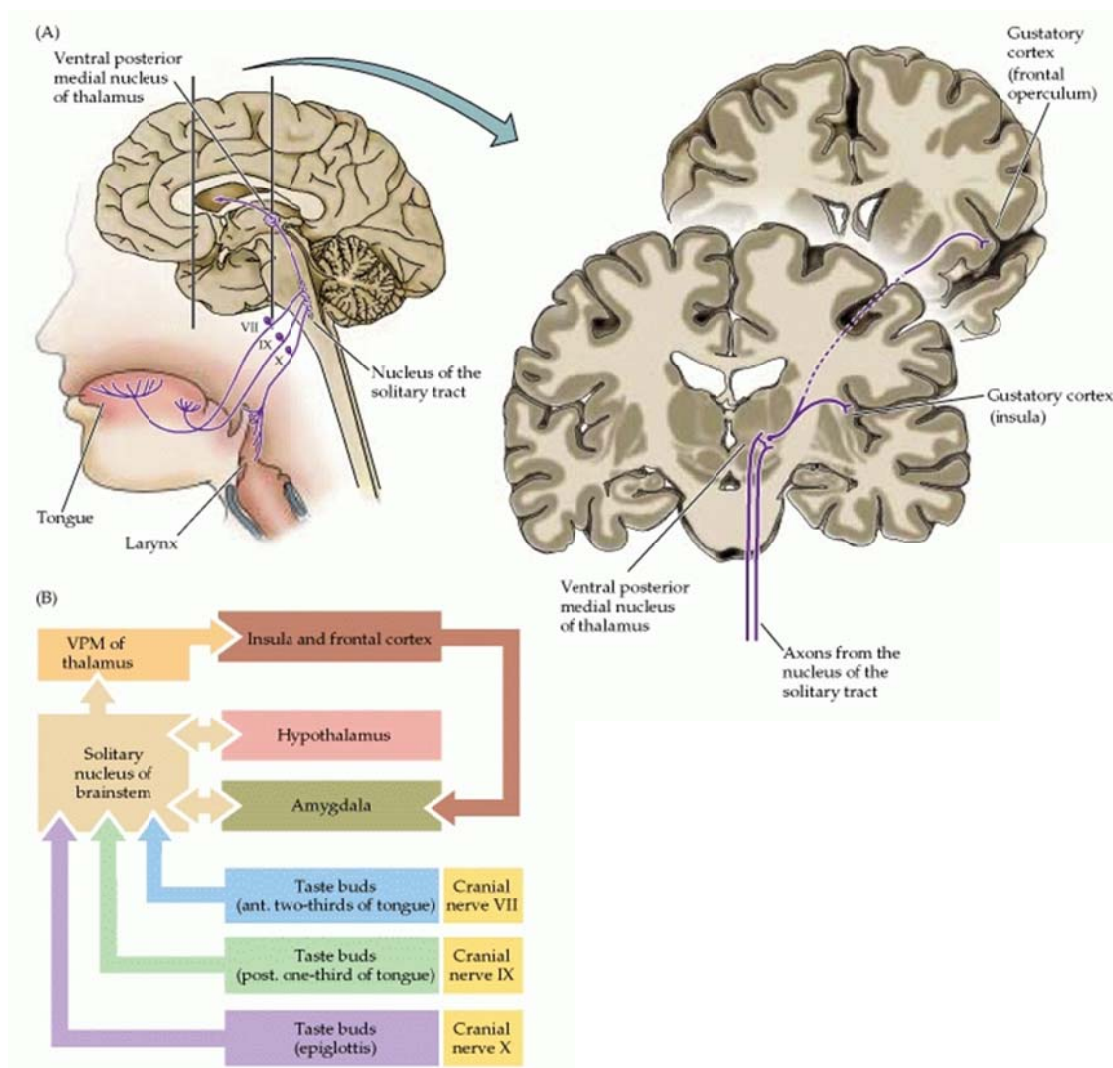


Figure 1.2 Organization of the taste system. (A) The relationship between receptors in the oral cavity and upper alimentary canal, and the nucleus of the solitary tract in the medulla (left). The coronal section shows the ventral posterior medial nucleus of the thalamus and its connection with gustatory regions of the cerebral cortex (right). (B) Diagram of the basic pathways for processing taste information. (28)

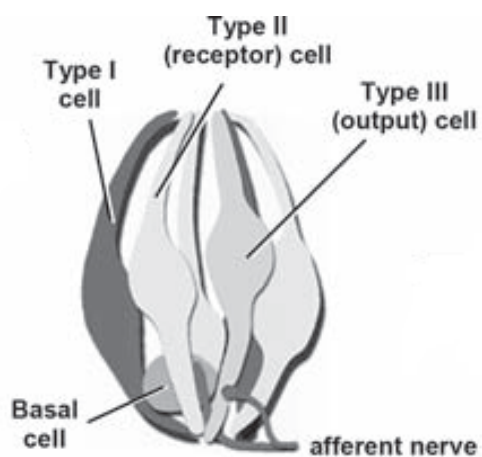


Figure 1.3 A schematic diagram of a mammalian taste bud and different taste cell types. Basal cells are proliferative cells. Type I taste cells are supporting cells. Type II taste cells are receptor cells for sweet, bitter and umami. Type III taste cells form synapses onto afferent nerve fibers, which receive and integrate information from other taste cells and release neurotransmitters. (39)

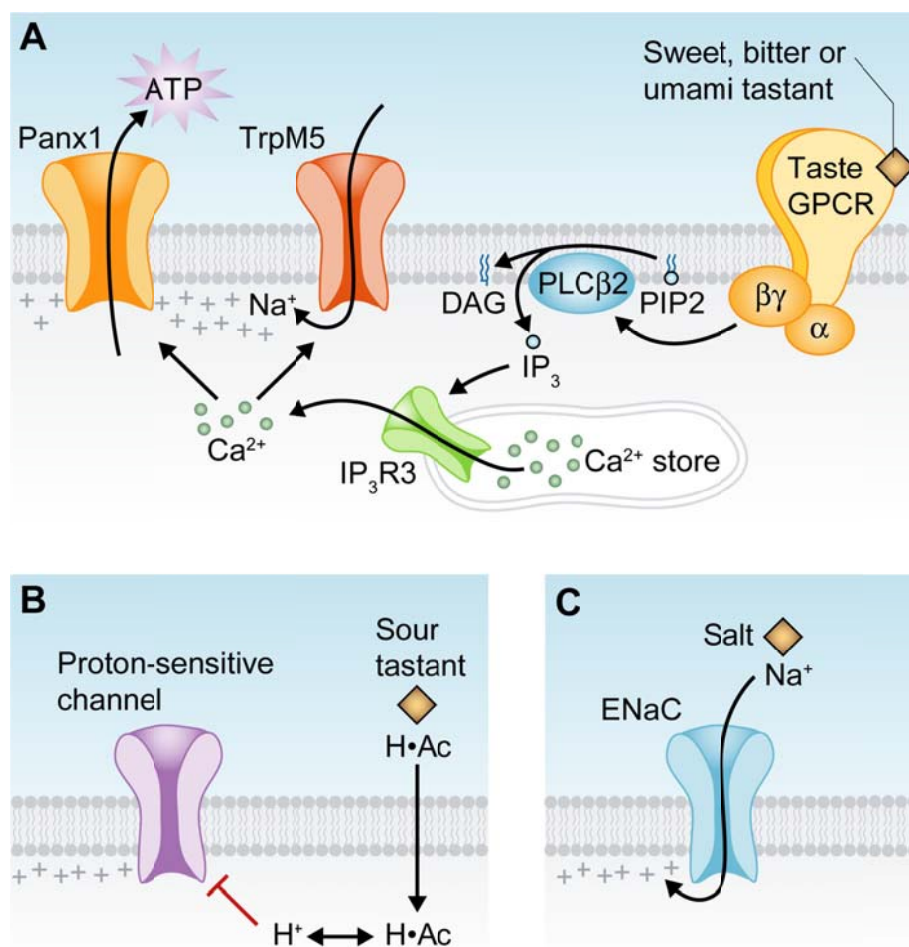


Figure 1.4 Molecular mechanisms for taste transduction. (A) In Type II taste cells, sweet, bitter, and umami compounds bind to GPCRs and activate the G protein-PLC pathway that elevates intracellular calcium level and depolarizes the cell via TrpM5 channels. The combined action of the elevated calcium and the membrane depolarization opens the gap junction hemichannels, causing ATP release into the extracellular space. (B) In Type III taste cells, organic acids (HAc) permeate through the plasma membrane and acidify the cytoplasm. Intracellular protons block a proton-sensitive potassium channel, leading to the depolarization of the cell. The opening of VGCCs elevates intracellular calcium level which in turn triggers the release of neurotransmitters. (C) The salty taste is detected by influx of sodium ions through ENaC channels on the apical membrane, which depolarizes the membrane and triggers the downstream signaling pathway. (81)

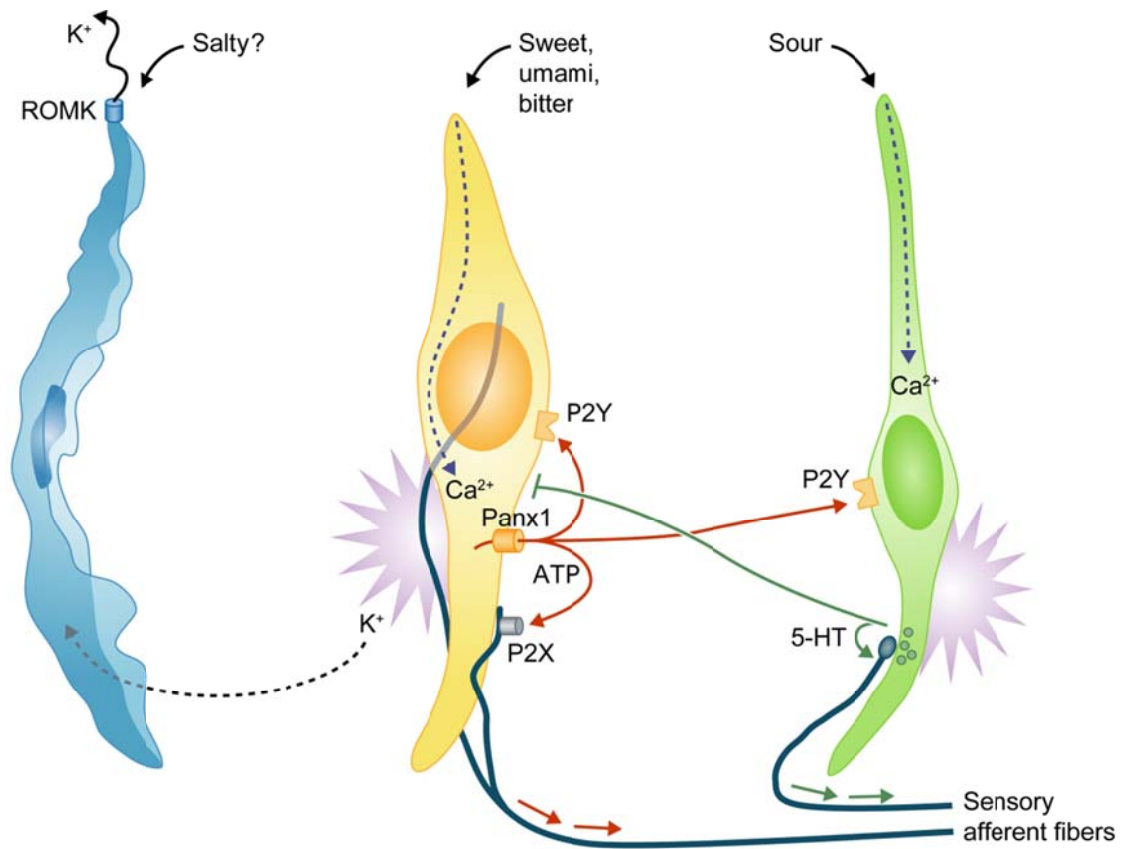


Figure 1.5 Cell-to-cell information processing in taste buds. Type I taste cells (blue), which may be directly involved in salty taste, clear out extracellular neurotransmitters and potassium ions. Type II taste cells release ATP through hemichannels (Panx1) when activated by sweet, bitter, and umami compounds. The secreted ATP excites adjacent nerve fibers, type II taste cells that release it, and type III taste cells which in turn release serotonin (5-HT) onto afferent nerves. 5-HT can also inhibit type II taste cells. (81)



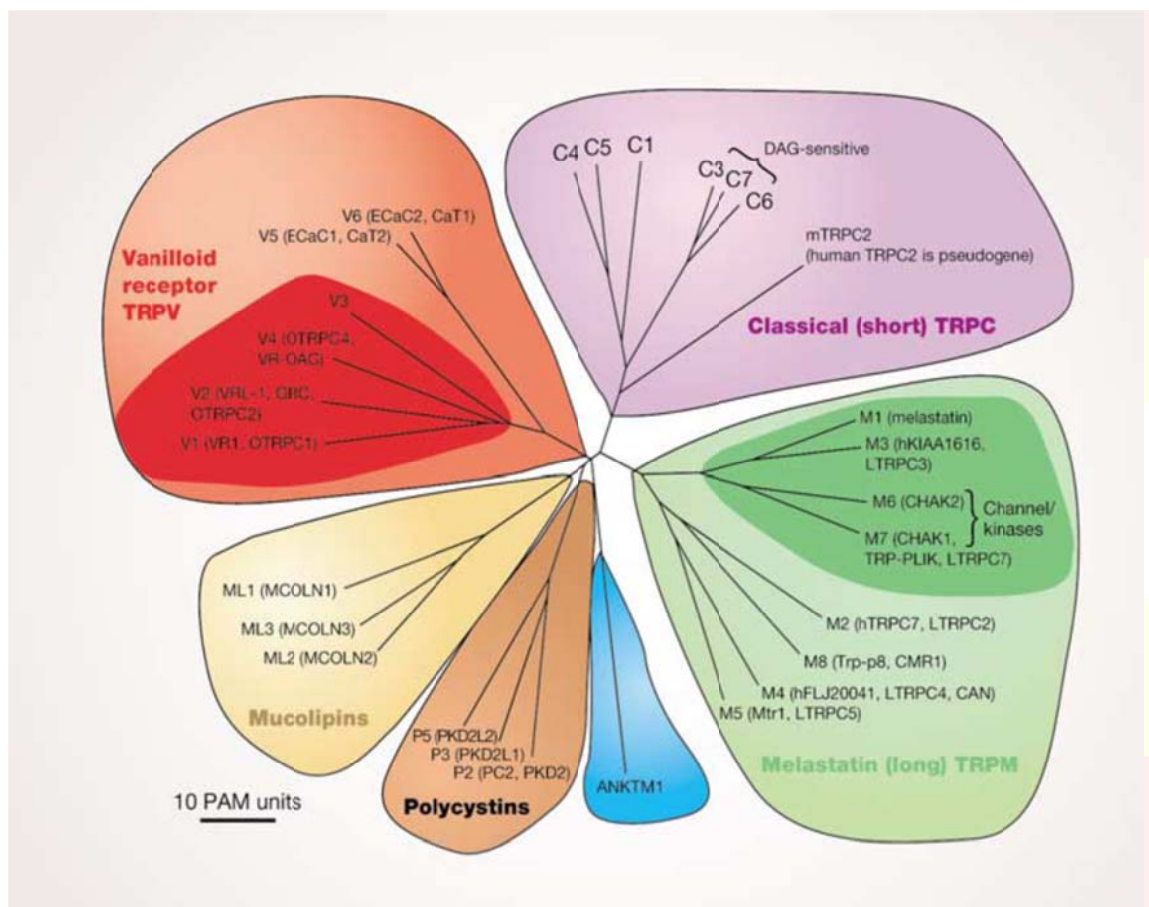


Figure 1.6 Mammalian TRP family tree. The evolutionary distance is shown by the total branch lengths in point accepted mutations units, which is the mean number of substitutions per 100 residues. (101)

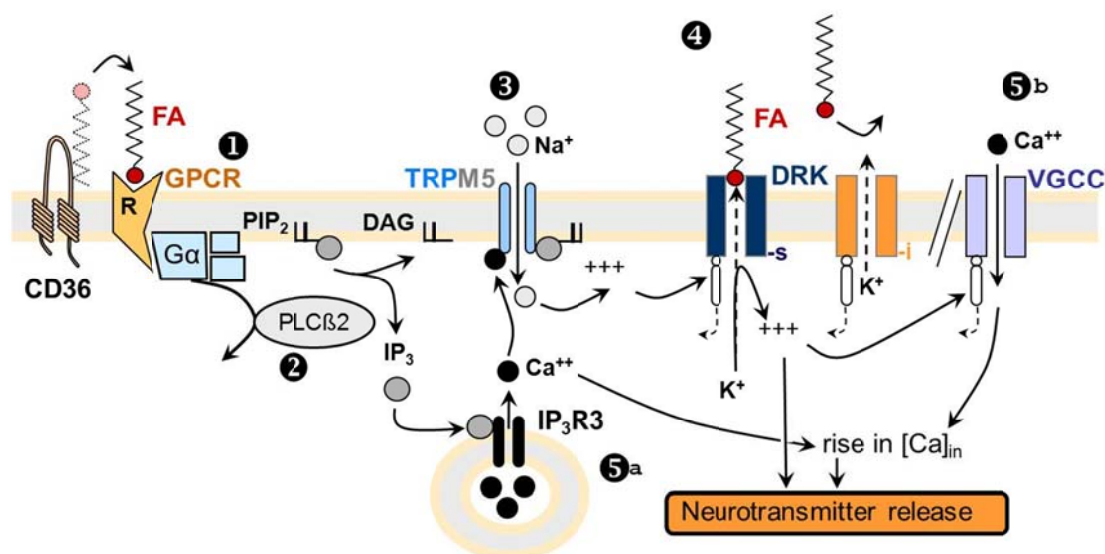


Figure 1.7 Hypothesized putative fat transduction pathway in taste cells. Fatty acids bind to GPCRs and activate G proteins that stimulate PLC $\beta$ 2, which in turn catalyzes the reaction to generate DAG and IP<sub>3</sub>. IP<sub>3</sub> binds to its receptors on the endoplasmic reticulum and releases calcium. The direct binding of calcium ions or the depletion of intracellular calcium stores activates TRPM5 channels whose activation allows the influx of sodium and further depolarizes the cell. This depolarization opens DRK channels. Inhibition of fatty acid-sensitive DRK channels by fatty acids further reinforces and prolongs the depolarization. This depolarization opens VGCCs leading to a calcium rise in the cytosol. The combined action of depolarization and elevated cytoplasmic calcium level eventually causes the release of neurotransmitters. FA: fatty acid; GPCR: G protein-coupled receptor; TRPM5: transient receptor potential channel type M5; DRK: delayed rectifying potassium channel; VGCC: voltage-gated calcium channel.

## CHAPTER 2

### TRANSIENT RECEPTOR POTENTIAL CHANNEL TYPE M5 IS ESSENTIAL FOR FAT TASTE

#### Abstract

Until recently, dietary fat was considered to be tasteless and its primary sensory attribute was its texture (1, 2). However, a number of studies have demonstrated the ability of components in fats, specifically free fatty acids, to activate taste cells and elicit behavioral responses consistent with there being a taste of fat. Here I show for the first time that long chain unsaturated free fatty acid, linoleic acid (LA), depolarizes mouse taste cells and elicits a robust intracellular calcium rise via the activation of transient receptor potential channel type M5 (TRPM5). The LA-induced responses depend on G protein-phospholipase C (PLC) pathway indicative of the involvement of G protein-coupled receptors (GPCRs) in the transduction of fatty acids. Mice lacking TRPM5 channels exhibit no preference for and show reduced sensitivity to LA. Together, these studies show that TRPM5 channels play an essential role in fatty acid transduction in mouse taste cells and suggest that fat may reflect a *bona fide* sixth primary taste.

#### Introduction

Obesity is one of the most serious health concerns in the western world and it is a disease that is reaching epidemic proportions. Being obese increases the incidence of no less than 30 diseases including diabetes, cardiovascular disease, cancer, and end-stage

renal disease. One of the precipitating factors that have been suggested to be linked to the increase in dietary-induced obesity is an increase in dietary fat intake (3-5). Despite this relationship, there is little information regarding the nature of the underlying mechanisms the body uses to recognize the sensory cues in fat. Obviously, understanding the mechanism that enables fat-responsive tissues, including the taste system, to recognize and respond to dietary fat would be of utmost importance in the fight to control fat intake and with it to reduce the incidence of dietary-induced obesity worldwide.

Until recently, dietary fat was considered to be tasteless and it was assumed that its primary sensory attribute was its texture (1, 2), primarily detected through the somatosensory system. However, data from our laboratory and others support the idea that in addition to its texture, fat may also be a basic taste that is capable of activating the gustatory system, specifically taste receptor cells in the oral cavity. Gilbertson *et al.* provided the first evidence that free fatty acids activate taste cells by inhibiting delayed rectifying potassium (DRK) channels (6-8). More recently, several additional fatty acid-responsive proteins have been identified that may play a role in initiating fatty acid transduction, including the fatty acid binding protein, CD36 (9-12) and several GPCRs (13-18). A very recent report showed that GPR120 null and GPR40 null mice exhibited a diminished preference for and diminished taste nerve responses to several fatty acids, strongly suggesting that GPR40 and GPR120 may play a role as the primary receptors for the taste of fat (18). Thus it is reasonable to hypothesize that fatty acids may activate taste cells like the other “traditional” taste qualities (sweet, bitter, umami, salty, and sour), which eventually leads to the release of neurotransmitters.

In this study, I show that the prototypical polyunsaturated long chain free fatty acid, LA, depolarizes taste cells and elicits robust intracellular calcium rise. The LA-induced responses are significantly reduced when the activities of G proteins and PLC are inhibited, suggesting the involvement of G protein-PLC pathway in the fatty acid transduction. LA activates taste cells through the activation of a monovalent cation-selective channel TRPM5. When TRPM5 is inhibited by its specific blocker, or deleted from the genome, LA-induced responses are significantly reduced. Mice lacking TRPM5 channels show no preference for and reduced sensitivity to LA, which further confirms the role TRPM5 channel plays in fatty acid transduction. Together, these studies elucidate possible fat taste transduction pathway in mouse taste cells, and demonstrate the critical role TRPM5 channel plays in this process.

## Materials and Methods

### **Animals**

The TRPM5 knockout strain has been described in detail previously (19). All experiments were performed on adult (2-6 month) male C57BL/6J or TRPM5 knockout mice that were maintained on a 12-h:12-h day/night cycle with normal mouse chow and water provided ad libitum. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Utah State University and were performed in accordance with American Veterinary Medical Association guidelines.

### **Taste Cell Isolation**

Individual taste buds or taste cells were isolated from the tongues using techniques previously described (6-8). Briefly, tongues were removed and immediately immersed in ice-cold Tyrode's solution. The anterior portion of the tongue containing the fungiform papillae was injected between the muscle layer and the lingual epithelium with approximately 0.2 ml of physiological saline (Tyrode's) containing a mixture of collagenase I (1 mg/ml; Roche Applied Science, Indianapolis, IN), dispase II (2.45 mg/ml; Roche Applied Science, Indianapolis, IN), and trypsin inhibitor (1 mg/ml; type I-S; Sigma-Aldrich, St Louis, MO). Between 0.2 and 0.3 ml of the same enzyme solution was also used to inject the area surrounding the two foliate papillae and the circumvallate papilla. The injected tongue was incubated in Tyrode's and bubbled with O<sub>2</sub> for 45 min at room temperature (RT). Following incubation, the tongue was washed with saline. The lingual epithelium was removed from the underlying muscle layer with forceps, pinned out in a Sylgard-lined petri dish containing the same enzyme solution, and incubated for appropriately 7 min. After incubation, the lingual epithelium was washed with saline and incubated in the calcium-magnesium free Tyrode's for 5 min. Individual taste cells were moved from the epithelium under low magnification ( $\times 50$ ) by gentle suction from a fire-polished pipette ( $\sim 100$   $\mu$ m pore) and plated immediately into a recording chamber containing Tyrode's for patch clamp recording experiments, or onto a coverslip coated with Cell-Tak Cell and Tissue Adhesive (BD Biosciences, San Jose, CA) for calcium imaging, or into a 0.5 ml microfuge tube on ice for the reverse transcriptase PCR (RT-PCR) and quantitative real time PCR (qPCR) assays.

## Solutions

Standard extracellular saline solution (Tyrode's) contained (in mM): 140 NaCl, 5 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. Calcium free saline (calcium free Tyrode's) contained (in mM): 140 NaCl, 5 KCl, 1 EGTA, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. Sodium free saline (sodium free Tyrode's) contained (in mM): 280 mannitol, 5 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose; pH 7.40 adjusted with TrisOH; 310 mOsm (adjusted with mannitol). 60 mM sodium Tyrode's contained (in mM): 50 NaCl, 180 mannitol, 5 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, 10 Na pyruvate; pH 7.40 adjusted with TrisOH; 310 mOsm (adjusted with mannitol). 10 mM sodium Tyrode's contained (in mM): 280 mannitol, 5 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, 10 Na pyruvate; pH 7.40 adjusted with TrisOH; 310 mOsm (adjusted with mannitol). Calcium-magnesium free saline (calcium-magnesium free Tyrode's) contained (in mM): 140 NaCl, 5 KCl, 2 BAPTA, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. A potassium-based intracellular solution was used for measurement of membrane potential contained (in mM): 140 K gluconate, 1 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10 HEPES, 11 EGTA, 3 ATP, and 0.5 GTP; pH 7.2 adjusted with KOH; 310mOsm. A cesium-based intracellular solution was used for recording TRPM5 currents and contained (in mM): 140 Cs acetate, 1 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10 HEPES, 11 EGTA, 3 ATP, and 0.5 GTP; pH 7.2 adjusted with TrisOH; 310 mOsm. U73122, an inhibitor of PLC, and U73343, the inactive analog of U73122 were purchased from Sigma (St. Louis, MO). GDP- $\beta$ -S, an inhibitor of G protein activation

was obtained from EMD Biosciences (La Jolla, CA). Thapsigargin, an inhibitor of sarco/endoplasmic reticulum calcium ATPase was purchased from MP Biochemicals (Solon, OH). TPPO, an inhibitor of TRPM5 channels was purchased from Maybridge (Tintagel, UK). 9-phenanthrol, an inhibitor of TRPM4 channels was purchased from Sigma (St. Louis, MO). LA was made as stock solutions (25 mg/ml) in EtOH, evacuated with N<sub>2</sub> and stored at -20 °C for up to 2 weeks until they were diluted for use immediately prior to the experiment. Hanks' buffered salt solution with HEPES buffer and Na pyruvate with fetal bovine serum (HHP with 2% FBS) contained: 98% 1× HBSS, 1% HEPES, 1% Na pyruvate, and 2% heat-inactivated fetal bovine serum; stored at 4°C for up to 1 week. Tastant mixture contained: 20 mM saccharin, 100 μM SC45647, 10 mM denatonium benzoate, 100 μM cycloheximide, and 5 mM monosodium glutamate. 100 mM KCl solution contained (in mM): 45 NaCl, 100 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with KOH; 310 mOsm.

### **Patch Clamp Recording**

Recordings were made from individual taste cells or taste cells maintained in the taste bud in the whole-cell variation of the patch clamp technique (6-8). Membrane potential (current clamp mode) and LA-induced currents (voltage clamp mode) were measured with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA). Borosilicate pipettes were pulled on a Sutter P-97 puller (Sutter Instruments, Novato CA) and subsequently fire polished on a microforge (model MF-9; Narishige, East Meadow NY) to a resistance of 5-10 MΩ. Series resistance and cell capacitance were compensated optimally before the recording. Commands were delivered and data were recorded with



pCLAMP software (version 10, Molecular Devices, Sunnyvale, CA) interfaced to an AxoPatch 200 B amplifier with a Digidata 1322 A A/D board (Axon Instruments, Union City, CA). Data were collected at 2-5 kHz and filtered on-line at 1 kHz. For membrane potential measurement, LA was applied by bath application, and the membrane potential of taste cells was recorded continuously before, during and after LA application using the current clamp mode of the amplifier while holding the cell at its zero current level (i.e. at rest). To determine the ionic dependence of LA-induced changes in membrane potential, membrane potential was recorded in three different extracellular solutions including Tyrode's, sodium free Tyrode's and calcium free Tyrode's. LA-induced TRPM5 currents in taste cells were recorded using the voltage clamp mode. Typical inward currents were recorded at a holding potential of -100 mV. LA was applied focally from a pipette positioned near the cell and delivered by a PicoSpritzer III (Parker Hannifin Corp, Cleveland, OH) controlled by the data acquisition and analysis software. Ramp protocols from -100 mV to +100 mV (500 ms duration) were used to generate instantaneous current-voltage (I-V) relationship of LA-induced TRPM5 current in various solutions. Data were analyzed by an unpaired, two-tailed Student's t-test (Figure 2.2D, 2.3C), or one-way ANOVA followed by Bonferroni's post hoc analysis (Figure 2.4E, J, 2.5D, 2.7C). Significance was set at  $\alpha = 0.05$  for all the analysis.

### **Calcium Imaging**

Single taste cells were loaded with Fura-2AM (5  $\mu$ M; Molecular Probes, Eugene, OR) for 1 h in HHP with 2% FBS at room temperature in the dark. The coverslips were then mounted onto an imaging chamber (RC-25F, Warner Instruments, Hamden, CT),

placed on an inverted microscope (Nikon, Eclipse TE2000-S, Japan) and perfused continuously with Tyrode's. Cells were illuminated with a 100-watt xenon lamp and excitation wavelengths (340/380 nm) were delivered by a monochromator (Bentham FSM150, Intracellular Imaging Inc., Cincinnati, OH) at a rate of 20 ratios per minute. Fluorescence was measured by a CCD camera (DVC-340M, DVC Company, Austin, TX) coupled to a microscope and controlled by imaging software (Incyt Im2™, Intracellular Imaging). The ratio of fluorescence (340 nm/380 nm) was directly converted to calcium concentrations using a standard curve generated for the imaging system using Fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR). LA and other compounds were applied extracellularly with a bath perfusion system at a flow rate of 4 ml/min permitting complete exchange of the extracellular solution in less than 20 s. I recorded the resting calcium baseline for at least 30 s before each stimulus. My criteria for accepting calcium responses for analysis were that (1) responses could be elicited repetitively in the same cell by the same stimulus and (2) the peak response was at least ten times the variance of baseline fluctuation. For generation of concentration–response curves, some taste cells were treated with LA in an ascending concentration series, while others were tested in random order. No differences were seen using these two methods. Data were analyzed by a paired (Figure 2.1G, 2.5F) or unpaired (Figure 2.7E) two-tailed Student's t-test and significance was set at  $\alpha = 0.05$ .

### **Behavioral Assays**

A 48-h, two-bottle preference test was performed to examine whether the deletion of TRPM5 alters preference for LA. Procedures for the 48-h, two-bottle preference test

have been described in detail previously (8, 11, 18). Two groups of 8 mice each (1 group of wild-type male mice and 1 group of TRPM5 null male mice) were tested in this paradigm. Briefly, each group underwent a preference tests using LA (30  $\mu$ M) versus water. The concentration of LA chosen was consistent with the concentration of LA tested in my electrophysiological experiments. After each 24-h period, fluid intake was measured, bottles were replaced with fresh solutions, and the side (left versus right) of the test solution was altered to compensate for any innate side preference. Preference ratios were calculated as the amount of test solution intake in 48 h divided by the amount of water intake over the same period. Thus, a preference ratio of 0.5 indicates the test solution was neither preferred nor avoided relative to water. Differences between the two groups were analyzed for statistical significance using an unpaired, two-tailed Student's t-test and significance was set at  $\alpha = 0.05$  (Figure 2.8A).

A conditioned taste aversion (CTA) assay was performed to test whether the deletion of TRPM5 alters the ability to detect LA. In the CTA experiments, two groups of mice (1 group of wild-type male mice and 1 group of TRPM5 null male mice) were used. Each group was further assigned to categories to receive either LiCl (experimental manipulation, CTA) or saline (control) injections during testing in the following sample sizes: wild-type male LiCl,  $n = 7$ ; wild-type male NaCl,  $n = 3$ ; TRPM5 null LiCl,  $n = 9$ ; TRPM5 null male NaCl,  $n = 5$ . Details of the CTA behavioral tests have been described previously (20). Briefly, mice had ad libitum access to water until 24 h prior to conditioning and testing at which time the mice were placed on a 23.5-h water restriction schedule for the duration of the experiment. Two hours after the

training/conditioning/testing on each of the water restriction days, all mice were given 30-min access to water. All unconditioned stimulus (US) injections were dose dependent on body weight. All taste stimuli were mixed daily from reagent grade chemicals and presented at room temperature. Fatty acid stimulus concentrations were selected to be similar to concentrations previously shown to activate taste cells (8), and the conditioned stimulus (CS) of 100  $\mu$ M LA was selected based on preliminary behavioral data in our lab (not published). In addition to water, there were 10 test stimuli consisting of 0.1, 0.3, 1, 3, 10, 30 and 100  $\mu$ M LA, 100 mM sucrose, 3 mM denatonium benzoate, and 100 mM NaCl. Taste aversions were conditioned through two consecutive daily pairings of the CS and the US. At 9:00 AM on each conditioning day, mice first received a 1-ml intraoral application of the CS solution. Immediately after the intraoral application, the US was administered through intraperitoneal injections (20 ml/kg body weight dosage) of 150 mM LiCl to induce gastric distress or 150 mM NaCl (saline) as a control condition. All mice receiving a LiCl injection showed behavioral signs of gastric malaise, the unconditioned response, within 20 min of the injection. Two hours after the conditioning, all mice were given 30-min access to water. All testing was conducted in a MS-160 Davis Rig gustatory behavioral apparatus. A fan was located near the chamber in order to direct constant airflow along the longitudinal axis of the stimulus delivery tray serving to reduce olfactory cues for any given stimulus. Mice were trained to lick during water stimulus trials in the Davis Rig for three consecutive days prior to the initial conditioning day. Following the second conditioning day, two consecutive days of testing in the Davis Rig assessed the formation of conditioned and generalized taste aversions. Each daily test

session consisted of two blocks of 12 trials with stimulus durations of 5 s, wait times for the first lick of 150 s, and inter-stimulus intervals of 2 s. Each block included 1 trial of each test stimulus and two trials of water stimuli. The stimulus order within each block was randomly assigned. Total number of licks per stimulus was summarized across the two trials per test session and normalized using a lick ratio (licks per test stimulus/licks to water) in order to account for individual variances in the water-restricted motivation across the mice. All mice included in the data analysis sampled each stimulus at least once during each daily test session. Trials in which the mouse did not lick were excluded from analysis. Differences between LiCl and saline-injected mice within each group were analyzed for statistical significance using an unpaired, two-tailed Student's t-test and significance was set at  $\alpha = 0.05$  (Figure 2.8B).

### **RT-PCR and qPCR**

For RT-PCR checking expression of putative fatty acid-responsive proteins in taste bud cells, RNA was isolated using RNeasy® Micro Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. First-strand cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). The maximum volume of taste RNA or 50 ng of kidney RNA was used for the reaction in a total volume of 20  $\mu$ l. Reactions were also set up in which the reverse transcriptase enzyme was omitted as a control to detect genomic DNA contamination. After first-strand synthesis, 1  $\mu$ l of cDNA were added to a PCR mixture [final concentration: 1  $\times$  EconoTaq reaction buffer, 200  $\mu$ M 2-deoxynucleotide 5'-triphosphate (dNTP), 500 nM forward and reverse primers, and 1.25 U of *EconoTaq* polymerase]. Primers for GPR-40, -41, -43, -120,

Kv1.5, and TRPM4 were designed using Oligo 6.0 Primer Analysis Software (Molecular Biology Insights, Inc., Cascade CO). The primer sequences and control tissues are listed in Table 2.1. Amplification using regular PCR included an initial 5-min denaturation step followed by 40 cycles of a three-step PCR: 15-sec denaturation at 95°C, 30-sec annealing at 60°C, 45-s extension at 72°C, and concluding with a 7-min final extension step. Amplified sequences were visualized using electrophoresis in 2% agarose gels poured using 1 TAE buffer (40 mM Tris-acetate and 1 mM EDTA).

For qPCR, taste cells were stored in TRI Reagent (MRC, Cincinnati, OH) for immediate extraction of RNA. Extraction was done according to manufacturer's protocol. cDNA was synthesized using the Message*BOOSTER*<sup>TM</sup> cDNA Synthesis Kit for qPCR (EPICENTRE Biotechnologies, Madison, WI). Commercially available TaqMan® Gene Expression Assays (ABI, Carlsbad, CA) were then used to detect the expression of CD36, GPR84, and TRPM5. Control tissues are listed in Table 2.1.

## Results

### **LA Depolarizes and Elicits an Intracellular Calcium Rise in Taste Cells.**

To explore the mechanism that enables the taste system to recognize and respond to dietary fat, I have used cell-based approaches including both functional calcium imaging and patch clamp recording. I first loaded single taste cells isolated from both fungiform and circumvallate papillae with the ratiometric fluorescent dye Fura-2AM and measured the LA-induced intracellular calcium change. LA elicited a robust and reversible increase in intracellular calcium in taste cells (Figure 2.1A, C). I also used a

series of concentrations of LA (1, 3, 10, 30, and 100  $\mu\text{M}$ ) and generated the concentration-response curve with a Boltzmann function which showed that the  $\text{EC}_{50} = 13.7 \mu\text{M}$  ( $n = 105$ , Figure 2.1B). In the following experiments to study the fatty acid-activated pathway in great detail, I used 30  $\mu\text{M}$  LA as the fatty acid stimuli. At this concentration, LA induced significant but not maximum responses as is shown in Figure 2.1B. Moreover, the LA-induced intracellular calcium rise was repeatable with the same amplitude.

I also performed patch clamp recording experiments to explore the effect of LA on membrane potential of taste cells using the whole-cell current clamp configuration. Taste cells were held at zero current level to determine the resting membrane potential ( $-45 \sim -55 \text{ mV}$ ). In patch clamp recording experiments, LA, applied by bath perfusion, elicited a large and reversible depolarization of  $40.42 \pm 1.73 \text{ mV}$  ( $n = 14$ ; Figure 2.2A) in taste cells that followed a time course similar to the change in intracellular calcium.

To investigate the dependence of LA-induced intracellular calcium rise on extracellular calcium, intracellular calcium and extracellular sodium ions, I carried out a series of ion substitution experiments. In the absence of extracellular calcium, LA-induced intracellular calcium rise was significantly reduced ( $n = 486$ ;  $p < 0.001$ ; Figure 2.1E, G). To look at the contribution of calcium from intracellular stores to the overall calcium rise, thapsigargin, an inhibitor of sarco/endoplasmic reticulum calcium ATPase, was used to perfuse taste cells for 5~7 min to deplete the intracellular calcium stores. The incubation in thapsigargin induced a robust rise in resting calcium levels, indicating the possible activation of store operated calcium entry (SOCE) triggered by the depletion of

the endoplasmic reticulum (ER). The application of LA first caused a rapid decrease in the basal calcium concentration, probably due to its ability to inhibit SOCE (21), and then increased the intracellular calcium concentration gradually. The LA-induced intracellular calcium rise after thapsigargin treatment was significantly reduced ( $n = 152$ ;  $p < 0.001$ ; Figure 2.1F, G), consistent with the interpretation that calcium ions from both intracellular stores and extracellular environment contribute to the fatty acid responses in taste cells. Interestingly, removal of extracellular sodium ions also caused a significant decrease in the LA-induced intracellular calcium rise ( $n = 312$ ;  $p < 0.001$ ; Figure 2.1D, G).

To determine what cations contributed to the LA-induced membrane depolarization, I manipulated the concentrations of ions extracellularly. Removal of extracellular calcium ions did not have a significant effect upon the LA-induced depolarization ( $40.14 \pm 0.77$  mV;  $n = 5$ ;  $p = 0.164$ ; Figure 2.2B, D). On the contrary, when extracellular sodium ions were removed, LA-induced depolarization was significantly reduced ( $2.22 \pm 0.10$  mV;  $n = 5$ ;  $p < 0.001$ ; Figure 2.2C, D). These results suggest that sodium influx is necessary for LA-induced depolarization and that there may be an additional site for calcium entry downstream of sodium entry (depolarization).

### **LA Activates Sodium Dependent Inward Currents in Taste Cells.**

Furthermore, I found in whole-cell voltage clamp experiments (holding potential = -100 mV) that rapid and focal application of LA caused a fast inward current in individual taste cells ( $247.3 \pm 29.77$  pA;  $n = 6$ ; Figure 2.3A). When extracellular sodium



ions were removed, this LA-induced inward current was significantly reduced ( $37.84 \pm 3.35$  pA;  $n = 15$ ;  $p < 0.001$ ; Figure 2.3B, C).

To determine the ionic dependence of the LA-induced current, I performed a series of ion substitution experiments to investigate the permeability properties of this conductance. In standard conditions with concentrations of monovalent cations equal on both sides of the membrane, the current-voltage relationship of LA-induced inward current showed a reversal potential  $\approx 0$  mV ( $n = 7$ ), suggesting that LA activated a non-selective monovalent cation-permeable pathway. Under the standard conditions, the inward current was mainly carried by sodium ions and the outward current was mainly carried by cesium ions. Consistent with this interpretation, changes in the concentration of extracellular sodium ions ( $n = 11$  for 60 mM extracellular sodium concentration;  $n = 14$  for 10 mM extracellular sodium concentration) led to a corresponding shift in the reversal potential of LA-induced inward current, which can be closely predicted by the Goldman-Hodgkin-Katz (GHK) equation for sodium, potassium and cesium-permeable conductance with the assumption that all three ions have relatively equal permeability (Figure 2.3D, E).

### **LA-Induced Responses Depend on G protein-PLC Pathway.**

Recently, several GPCRs have been identified as putative receptors for free fatty acids (13-15) and their expression in taste tissues has been verified by RT-PCR (Table 2.2) and immunocytochemical experiments (16-18). A very recent report showed that GPR120 null and GPR40 null mice exhibited a diminished preference for LA and oleic

acid (OA), and diminished taste nerve responses to several fatty acids, strongly suggesting that GPR40 and GPR120 may play a role as the primary receptors for the taste of fat (18).

To further confirm the role of G proteins in the fatty acid transduction pathway, I used GDP- $\beta$ -S to reversibly block the activation of G proteins, and examined its effect on the LA-induced depolarization and inward current. Electrophysiological experiments showed that LA-induced depolarization ( $8.73 \pm 1.63$  mV;  $n = 7$ ;  $p < 0.001$ ) and inward current ( $48.21 \pm 5.78$  pA;  $n = 10$ ;  $p < 0.001$ ) were significantly reduced when the activation of G proteins was inhibited (Figure 2.4A, B, E, F, G, J). I examined the involvement of PLC in the fatty acid transduction pathway by using the PLC blocker U73122. In presence of U73122, the LA-induced depolarization ( $6.51 \pm 0.73$  mV;  $n = 11$ ;  $p < 0.001$ ) and inward current ( $25.24 \pm 3.32$  pA;  $n = 11$ ;  $p < 0.001$ ) were significantly reduced (Figure 2.4C, E, H, J), whereas the LA-induced depolarization ( $38.41 \pm 2.49$  mV;  $n = 10$ ;  $p < 0.001$ ) and inward current ( $224.74 \pm 23$  pA;  $n = 10$ ;  $p < 0.001$ ) were not affected when the taste cell was treated with its inactive analog U73343 (Figure 2.4D, E, I, J). My data suggest that LA-induced responses are downstream of the G protein-PLC pathway and support a pathway involving the activation of fatty acid-activated GPCRs (18).

#### **LA-Induced Currents in Taste Cells Are Carried Primarily Through TRPM5 Channels.**

During the past few years, the calcium-activated, monovalent cation-selective channel TRPM5 has been shown to play an essential role in the transduction of sweet,

bitter and umami tastes (22-26). However, tastant-induced TRPM5 currents have not been successfully recorded in native taste cells electrophysiologically. Since I have shown that the LA-induced inward current is monovalent cation-selective and downstream of PLC activation, I hypothesized that TRPM5 channels may be involved in this process. To test my hypothesis, I used the TRPM5 channel blocker triphenylphosphine oxide (TPPO) (27) to block the activity of TRPM5 channels, and examined its effect on the FA-induced responses in both calcium imaging and patch clamp recording experiments. TPPO treatment significantly reduced the LA-induced inward current ( $40.37 \pm 4.94$  pA;  $n = 12$ ;  $p < 0.001$ ) compared with control ( $260.34 \pm 29.8$  pA;  $n = 5$ ) (Figure 2.5A, B, D). To control for variances between cells, the functional output in calcium imaging experiments was based on response in TPPO compared to the response in normal Tyrode's in the same cell. The results showed that TPPO treatment significantly reduced the intracellular calcium rise ( $0.276 \pm 0.046$ ;  $n = 76$ ;  $p < 0.001$ ) (Figure 2.5E, F), consistent with the interpretation that TRPM5 channels are involved in the LA-activated signalling pathway.

Interestingly, the LA-induced inward current was not completely blocked by the TPPO treatment, suggesting either that TPPO does not completely block TRPM5 channels, or that there might be other or TRPM5-independent pathways involved in the LA-induced inward current. Since transient receptor potential channel type M4 (TRPM4) is also monovalent cation-selective and has very similar properties to TRPM5 channels (28) and it is apparently expressed in taste cells (Figure 2.6), I further tested if the residual current after TPPO treatment was carried by TRPM4 channels. I used TPPO and

9-phenanthrol (29), a TRPM4 channel blocker, together to block both TRPM5 and TRPM4 channels. Patch clamp recording data showed that the addition of 9-phenanthrol did not significantly reduce LA-induced current any further ( $26.96 \pm 2.86$  pA;  $n = 10$ ;  $p = 0.051$ ), suggesting that TRPM4 channels are not responsible for the small, residual LA-induced inward current (Figure 2.5C, D). Based upon its permeability properties, voltage-dependence and pharmacology, my results strongly suggest that LA-induced currents in taste cells are carried primarily through TRPM5 channels.

To directly assess the role of TRPM5 channels in the fatty acid signalling pathway, I used a transgenic mouse model with a TRPM5 gene deletion (19). Single taste cells were isolated from both fungiform and circumvallate papillae from mice lacking TRPM5. In these cells, LA-induced inward current was significantly reduced ( $23.33 \pm 2.61$  pA;  $n = 12$ ;  $n < 0.001$ ) (Figure 2.7A, C). In calcium imaging experiment, LA-induced intracellular calcium rise was significantly reduced as well ( $120.52 \pm 11.67$  nM;  $n = 67$ ;  $p < 0.001$ ) compared with the control ( $206.53 \pm 16.87$  nM;  $n = 49$ ) (Figure 2.7D, E). These results validated the involvement of TRPM5 channels in fatty acid transduction. Consistent with the findings in wild-type mice, the residual LA-induced inward current cannot be further reduced by blocking TRPM4 channels with 9-phenanthrol ( $9.99 \pm 1.84$  pA;  $n = 10$ ;  $p = 0.051$ ) (Figure 2.7B, C).

#### **Mice Lacking TRPM5 Channels Exhibit No Preference for and Show Reduced Sensitivity to LA.**

Recently, behavioral experiments have shown that wild-type mice preferred a LA emulsion to the vehicle in two-bottle tests (11). However, there are no reports

investigating the preference for fatty acid in TRPM5 null mice. I performed a 48-hour two-bottle preference test using both wild-type and TRPM5 null mice. The results showed that TRPM5 null mice were indifferent to a LA emulsion, whereas wild-type mice preferred LA to vehicle as expected (Figure 2.8A) (11). Based on this finding, I hypothesized that the TRPM5 null mice have lost their ability to respond to fatty acids. To test this hypothesis, I performed a series of brief-access (5 s) behavioral assays, which eliminated post-ingestive cues for fatty acids, following the formation of a conditioned taste aversion (CTA) to LA (conditioned stimulus: 100  $\mu$ M LA) (20). In the CTA assays, wild-type mice showed significant aversions at relatively low LA concentrations (3  $\mu$ M), whereas the TRPM5 null mice did not show any significant aversions at LA concentrations up to 100  $\mu$ M, suggesting their loss of sensitivity to LA at the concentrations tested (Figure 2.8B). These results validate the critical role TRPM5 channels play in the fatty acid transduction pathway.

## Discussion

As mentioned before, for a long time, it was widely accepted that fat was tasteless and its most salient cue was its texture (1, 2), which was usually described as “oiliness,” “creaminess,” “mouthfeel,” or “slipperiness.” In 1997, Gilbertson *et al.* provided the first direct evidence that fatty acids were very likely to elicit a gustatory (i.e., taste) cue in rat taste receptor cells, suggesting the presence of a sensory mechanism for fat in taste receptor cells (6). Using patch clamp recording, he showed that the essential fatty acids (*cis*-polyunsaturated fatty acids) activated taste receptor cells through the inhibition of

DRK channels (6). Since then, more evidence from both electrophysiological and behavioral experiments supported the idea that fatty acids are capable of activating taste receptor cells as open channel blockers of DRK channels (7, 8), which are known to be implicated in the transduction pathway of a variety of taste stimuli. However, one “problem” with this model is that only a small percentage of these DRK channels are open at resting membrane potentials, which indicates that there is an upstream signaling pathway that provides the depolarization needed to open DRK channels. Since several GPCRs have been identified as putative receptors for free fatty acids (13-15) and their expression in taste tissues has been verified (Table 2.2) (16-18), a possible resolution to this apparent confound is that free fatty acids activate these GPCRs, which activate a series of signaling cascades that result in the depolarization needed to open DRK channels which subsequently are inhibited by fatty acids, depolarizing the cell further.

In this study I first showed that LA was capable of depolarizing taste cells, which may provide the stimulus to open DRK channels. Since changes in intracellular calcium concentration have been considered to be one of the most important indicators of cellular activity, I also performed a series of calcium imaging experiments to explore the effect of LA on intracellular calcium changes. In the initial calcium imaging experiments, I found that removal of extracellular sodium or calcium ions significantly reduced LA-induced intracellular calcium rise, which suggested that sodium influx is required for the LA-induced intracellular calcium rise. Then in patch clamp recording experiments, I found that LA-induced depolarization was dependent on extracellular sodium ions but not on extracellular calcium ions. These patch clamp recording data in conjunction with the

calcium imaging data suggested that LA-induced depolarization is dependent on sodium entry and is required for the opening of DRK channels, which depolarize the cell further through inhibition by fatty acids and eventually open voltage-gated calcium channels (VGCCs) and allow the influx of calcium ions.

I also showed that LA initiated a rapid inward current in taste cells. This LA-induced inward current was significantly reduced when extracellular sodium ions were removed, consistent with the findings that LA-induced depolarization and intracellular calcium rise were dependent upon sodium influx as well. Then ion substitution experiments demonstrated that this LA-activated conductance revealed a monovalent cation-selective pathway. The subsequent experiments showed that blocking the activation of G proteins and PLC by GDP- $\beta$ -S and U73122, respectively, significantly reduced LA-induced responses, indicating the possible involvement of GPCRs in initiating fatty acid-activated signaling pathway.

During the past few years, the calcium-activated, monovalent cation-selective channel TRPM5 has been shown to play an essential role in the transduction of sweet, bitter and umami tastes (22-26). Since the LA-induced inward current is monovalent cation-selective and downstream of PLC activation, I investigated the possibility of TRPM5 involvement in LA-induced signalling pathway in taste cells. TPPO, the specific TRPM5 channel blocker, significantly reduced the LA-induced responses, suggesting that TRPM5 channels are very likely to be the channels activated by fatty acids and allowed the influx of sodium ions. This was the first time that tastant-induced TRPM5 current was successfully recorded in intact, native taste cells. Both patch clamp recording and calcium

imaging data using taste cells from TRPM5 null mice further verified the involvement of TRPM5 channels in the fat transduction pathway. Interestingly, LA-induced inward current cannot be completely blocked by TPPO, and 4-phenanthrol, the TRPM4 channels blocker, cannot reduce this residual current any further, suggesting that there might be additional fatty acid-responsive proteins or receptors involved, or there may exist parallel fatty acid-activated TRPM5-independent pathways in taste cells. Furthermore, I used a transgenic mouse model with a TRPM5 gene deletion. Compared with the wild-type mice, these TRPM5 null mice exhibited no preference for and showed reduced sensitivity to LA, validating the essential role TRPM5 channels play in the fat taste transduction. Taken together, my findings suggest that fatty acid-activated signaling pathway appears to involve fatty acid-activated GPCRs, G proteins, PLC, sodium influx through TRPM5 (necessary for depolarization), blocking of DRK channels by fatty acids, and calcium influx via VGCCs (activated by depolarization), as shown schematically in Figure 2.9.

Interestingly, a recent report (18) suggested that both GPR120, which is primarily expressed in fungiform and circumvallate taste receptor cells, and GPR40, expressed mainly in Type I taste cells from the circumvallate papillae, contribute to fatty acid signaling. My data would argue that GPR120 is more relevant to LA taste since GPR120 is expressed primarily in Type II cells (TRPM5-expressing), which do not apparently express GPR40. Loss of TRPM5 resulted in a complete inhibition of preference for LA and the ability to form a CTA against this fatty acid further, which suggests that Type II taste cells might be the primary receptor cells for linoleic acid. Since the ducts of Von Ebner's glands can secrete highly active lingual lipase, the enzyme responsible for



efficient free fatty acid release from dietary triglycerides and in the oral cavity capable of hydrolyzing ~70  $\mu\text{mol}$  of triglycerides per minute, it has been reported that free fatty acids produced from dietary fat can reach a concentration high enough to stimulate taste cells (30). Since the concentration-response curve showed the  $\text{EC}_{50} = 13.7 \mu\text{M}$ , which resided in the normal concentration range of free fatty acid in the oral cavity during dietary fat intake, these findings support the idea that fat may represent a sixth basic taste in addition to the five well accepted taste qualities: salty, sour, sweet, bitter and umami, and that TRPM5 channels are not only the key elements in sweet, bitter and umami taste, but also an essential player in the fat taste signalling pathway.

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Table 2.1 Primer sequences and control tissue for GPR-40, -41, -43, -84, -120, CD36, Kv1.5, TRPM5, and TRPM4. GPR84, CD36, and TRPM5 (marked in grey) were checked by TaqMan® Gene Expression Assays directly purchased from Applied Biosystems, (Carlsbad, CA).

Target	GeneBank Accession No.	Sense/Antisense primer	Corresponding nucleotide sequence	Control tissues
GPR40	NM_194057	5'-TTCTTTCTGCCCTTGGTTAT-3'	571-587	pancreas or STC-1
		5'-GCCCTGAGCTTCCGTTTG-3'	648-665	
GPR41	NM_029771	5'-GAGGATGATCTTCGCAGTG-3'	1384-1402	brain or STC-1
		5'-GGCTGCCAGGTTGACTATGT-3'	1530-1549	
GPR43	NM_146187	5'-GCGGGCATCAGCATAGAA-3'	836-853	heart or STC-1
		5'-CCCACCTGCTCGGTTGAGTT-3'	986-1005	
GPR84				brain or STC-1
GPR120	NM_181748	5'-CTGCACATTGGATTGGC-3'	627-643	STC-1
		5'-TCTGGTGGCTCTCGGAGTAT-3'	786-805	
CD36				brain
Kv1.5	NM_145983	5'-TGCAGATCCTGGGTAAGACC-3'	1475-1494	brain
		5'-ATTGTCTGCCTCTGCGAAGT-3'	1574-1593	
TRPM5				taste
TRPM4	NM_175130	5'-GAGGATCATGACCCGAAAGG-3'	735-754	taste
		5'-TTCACCTTGGGCGATGTC-3'	898-915	

Table 2.2 RT-PCR and qPCR showing the expression of the putative fatty acid-responsive proteins in taste bud cells. CD36, fatty acid-activated GPCRs (GPR-40, -41, -43, -84, and -120), and fatty acid-sensitive DRK channels (Kv1.5) are all expressed in three types of taste bud cells.

Taste Bud Cells	CD36	GPR40	GPR41	GPR43	GPR84	GPR120	Kv1.5
Fungiform	+	-	-	+	+	-	+
Foliate	+	-	+	+	+	+	+
Circumvallate	+	-	+	+	+	+	+

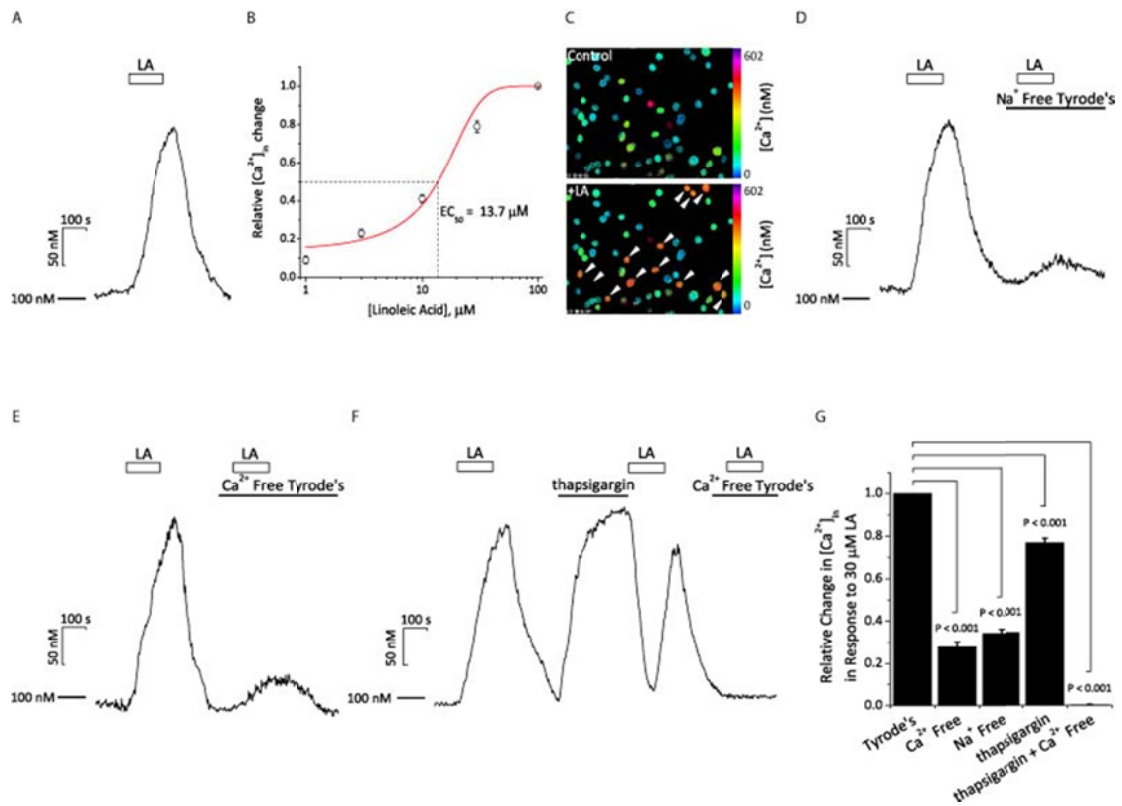


Figure 2.1 LA-induced intracellular  $Ca^{2+}$  rise was dependent on extracellular  $Na^+$ , extracellular  $Ca^{2+}$  and intracellular  $Ca^{2+}$  ions. LA was applied at a final concentration of 30  $\mu$ M. (A) Bath application of LA induced a robust intracellular  $Ca^{2+}$  rise in single taste cells. (B) Data from LA-induced changes in intracellular  $Ca^{2+}$  were fit with a Boltzmann function with an  $EC_{50} = 13.7 \mu$ M ( $n = 105$ ). LA was applied at 1-100  $\mu$ M final concentration. (C) Bath application of LA induced a robust intracellular  $Ca^{2+}$  rise in taste cells. Changes in intracellular  $Ca^{2+}$  concentration are pseudo-colored as depicted. Arrows indicate the taste cells that showed significant increases in intracellular  $Ca^{2+}$  concentration in response to LA. LA-induced intracellular  $Ca^{2+}$  rise in single taste cells in absence of extracellular  $Na^+$  ions (D), extracellular  $Ca^{2+}$  ions (E), and when intracellular  $Ca^{2+}$  stores were depleted with thapsigargin (F). (G) Mean responses (relative change in intracellular  $Ca^{2+}$  concentration)  $\pm$  S.E.M. to LA in Tyrode's (control),  $Ca^{2+}$  free Tyrode's ( $n = 486$ ),  $Na^+$  free Tyrode's ( $n = 312$ ), and when intracellular  $Ca^{2+}$  stores were depleted with thapsigargin ( $n = 152$ ).

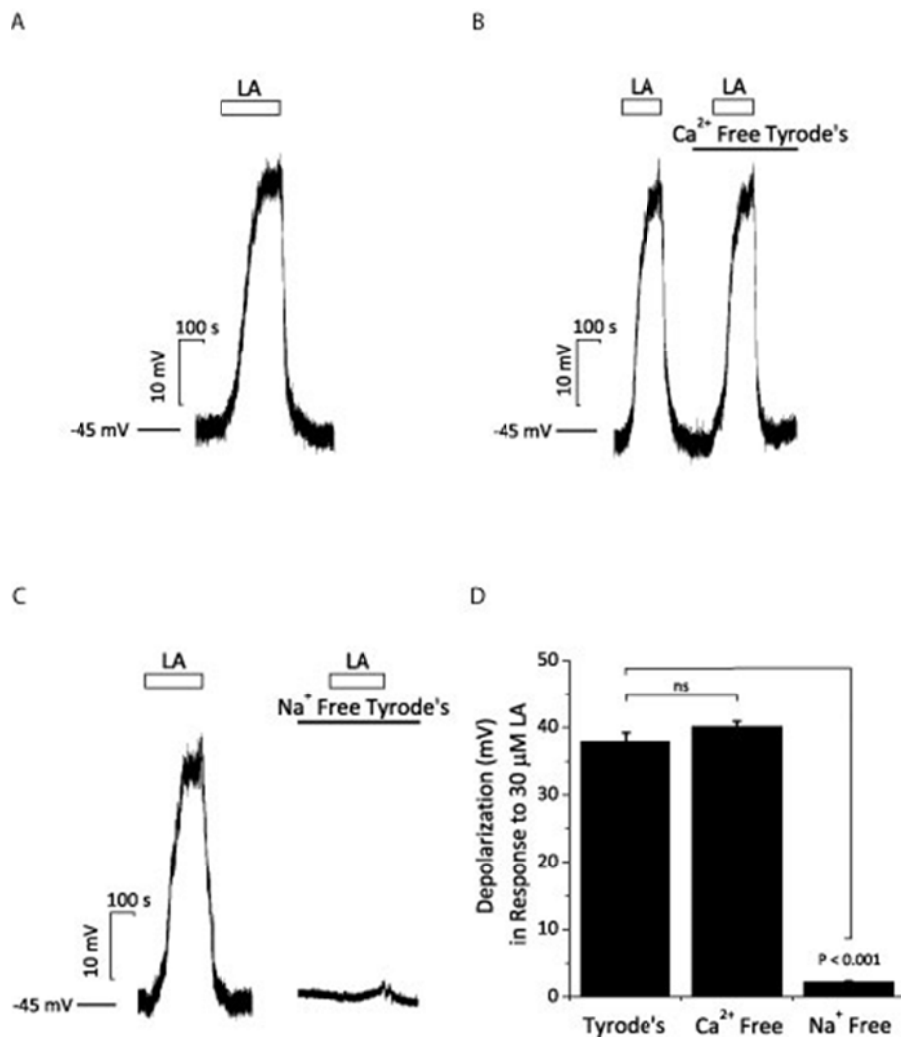


Figure 2.2 LA depolarized taste cells and this depolarization was dependent on extracellular  $\text{Na}^+$  ions but not on extracellular  $\text{Ca}^{2+}$  ions. LA was applied at a final concentration of 30  $\mu\text{M}$ . (A) Bath application of LA induced membrane depolarization in taste cells. Cells were held at 0 current level (e.g., at its resting membrane potential). LA-induced depolarization in taste cells in the absence of extracellular  $\text{Ca}^{2+}$  ions (B) and extracellular  $\text{Na}^+$  ions (C). (D) Mean responses (depolarization)  $\pm$  S.E.M. to LA in Tyrode's (n = 10),  $\text{Ca}^{2+}$  free Tyrode's (n = 5) and  $\text{Na}^+$  free Tyrode's (n = 5).



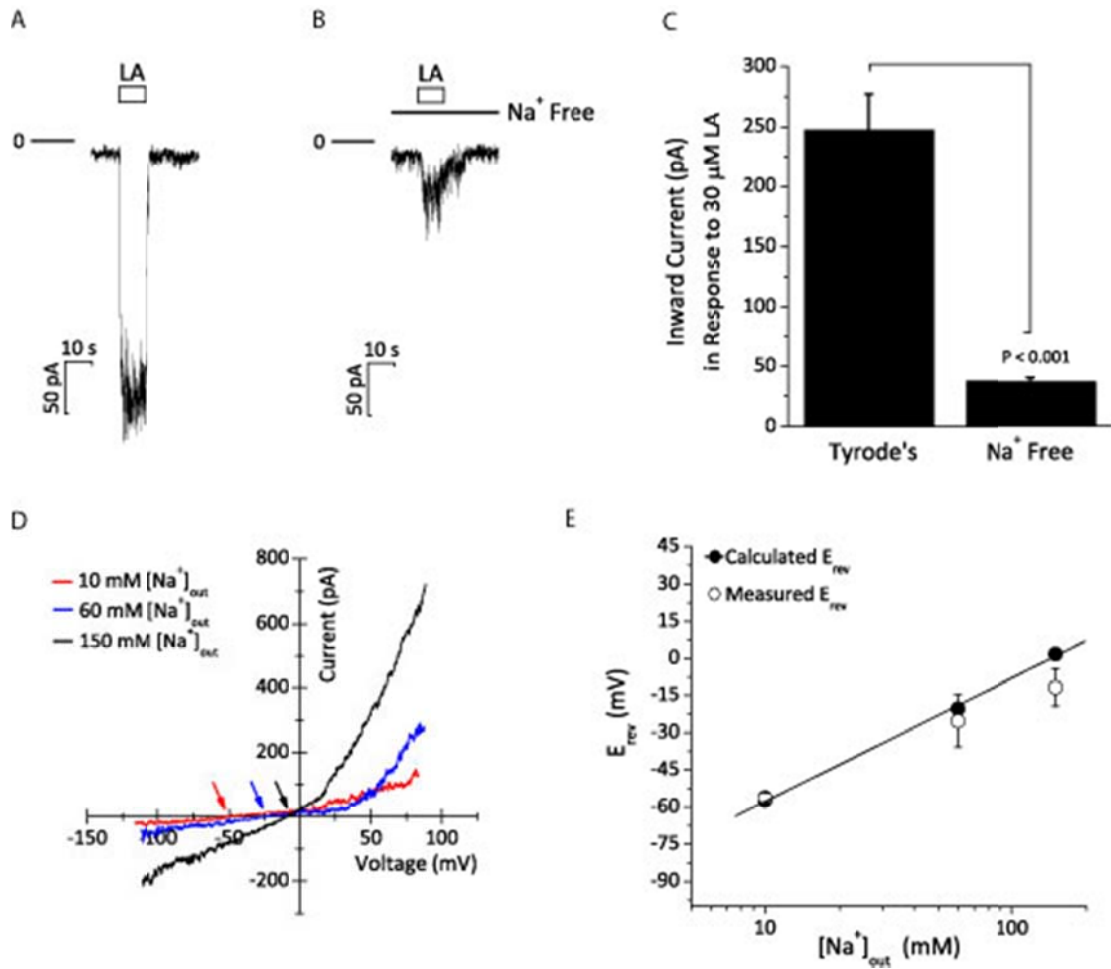


Figure 2.3 Rapid and focal application of 30  $\mu\text{M}$  LA elicited a monovalent cation-permeable current. LA-induced inward currents (holding potential = -100 mV) in taste cells in Tyrode's (A) and  $\text{Na}^+$  free Tyrode's (B). (C) Mean responses (peak inward current)  $\pm$  S.E.M. to LA in Tyrode's (control,  $n = 6$ ) and  $\text{Na}^+$  free Tyrode's ( $n = 15$ ). (D) Current-voltage relationship of LA-induced current at extracellular  $\text{Na}^+$  concentrations of 10, 60 & 150 mM. (E) Comparison between theoretical and measured reversal potentials  $\pm$  S.E.M. of LA-induced current at extracellular  $\text{Na}^+$  concentrations of 10 mM ( $n = 14$ ), 60 mM ( $n = 11$ ) & 150 mM ( $n = 7$ ). Reversal potentials (indicated by arrows) were corrected for liquid junction potential.

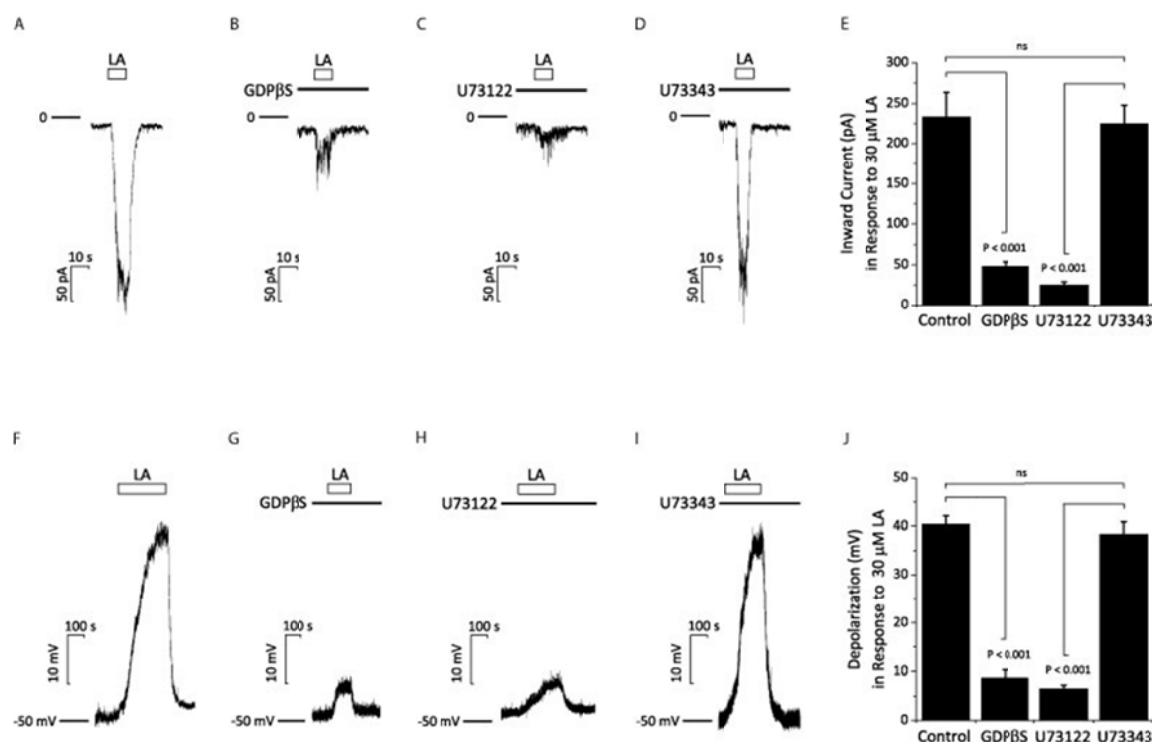


Figure 2.4 LA-induced responses were greatly reduced upon blocking the activity of G proteins and PLC. LA was applied at a final concentration of 30  $\mu$ M. Rapid and focal application of LA induced an inward current (holding potential = -100 mV) in control (A), GDP- $\beta$ -S (1 mM) treated (B), U73122 (3  $\mu$ M) treated (C), and U73343 (3  $\mu$ M, the inactive analog of U73122) treated (D) taste cells. (E) Mean responses (inward current)  $\pm$  S.E.M. to LA in control conditions (n = 5), with GDP- $\beta$ -S (1 mM) treatment (n = 10), with U73122 (3  $\mu$ M) treatment (n = 11), and with U73343 (3  $\mu$ M) treatment (n = 10). Current clamp studies in taste cells held at their 0 current level showing that LA induced membrane depolarization in control (F), GDP- $\beta$ -S (1 mM) treated (G), U73122 (3  $\mu$ M) treated (H), and U73343 (3  $\mu$ M, the inactive analog of U73122) treated (I) taste cells. (J) Mean responses (depolarization)  $\pm$  S.E.M. to LA in control conditions (n = 14), with GDP- $\beta$ -S (1 mM) treatment (n = 7), with U73122 (3  $\mu$ M) treatment (n = 11), and with U73343 (3  $\mu$ M) treatment (n = 10).

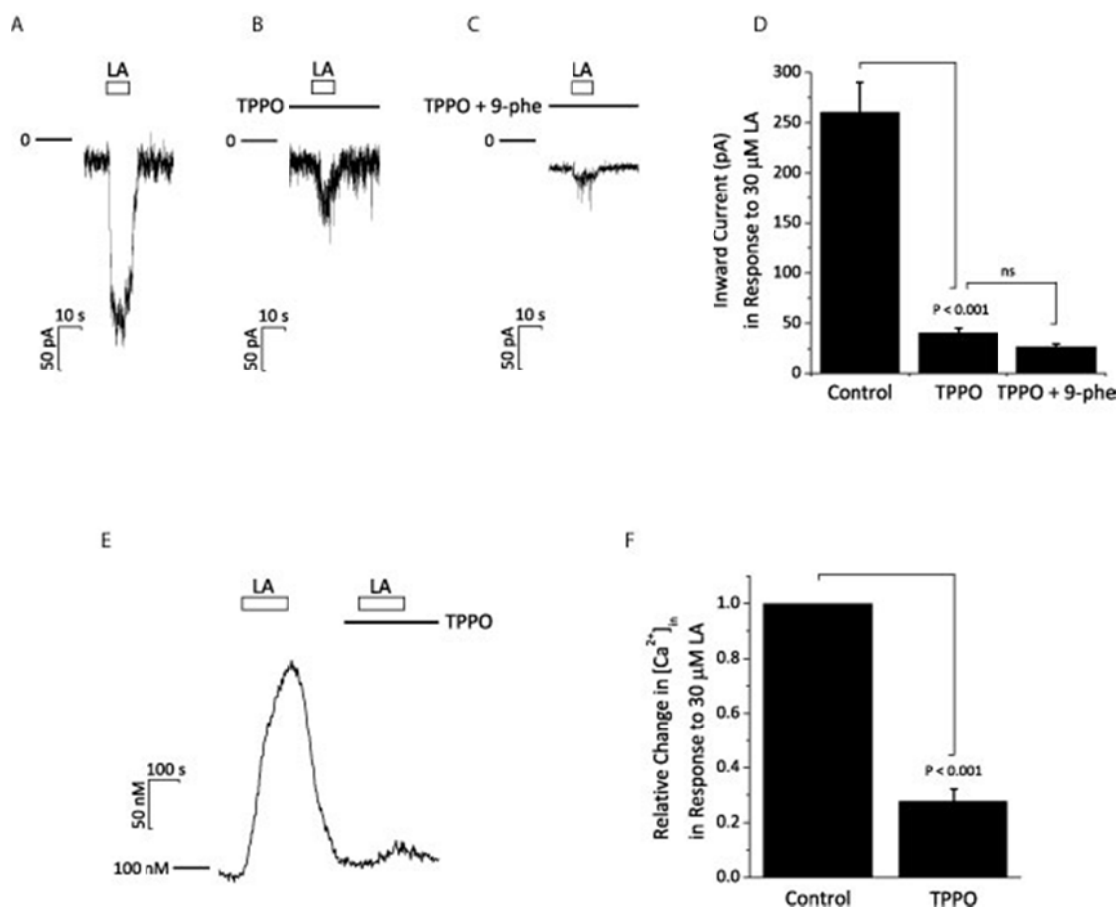


Figure 2.5 LA-induced responses were significantly reduced by the TRPM5 channel antagonist TPPO. LA (30  $\mu$ M)-induced inward currents in taste cells in Tyrode's (A), in presence of the TRPM5 antagonist TPPO (100  $\mu$ M) (B), and in presence of both the TRPM5 antagonist TPPO (100  $\mu$ M) and the TRPM4 antagonist 9-phenanthrol (100  $\mu$ M) (C). (D) Mean responses (peak inward current)  $\pm$  S.E.M. to LA in Tyrode's (control,  $n = 5$ ), with TPPO (100  $\mu$ M) treatment ( $n = 12$ ), and with both TPPO (100  $\mu$ M) and 9-phenanthrol (100  $\mu$ M) treatments ( $n = 10$ ). (E) LA (30  $\mu$ M)-induced intracellular  $Ca^{2+}$  rise in single taste cells in Tyrode's and with TPPO (100  $\mu$ M) treatment. (F) Mean responses (relative change in intracellular  $Ca^{2+}$  concentration)  $\pm$  S.E.M. to LA in Tyrode's (control) and with TPPO (100  $\mu$ M) treatment ( $n = 76$ ).

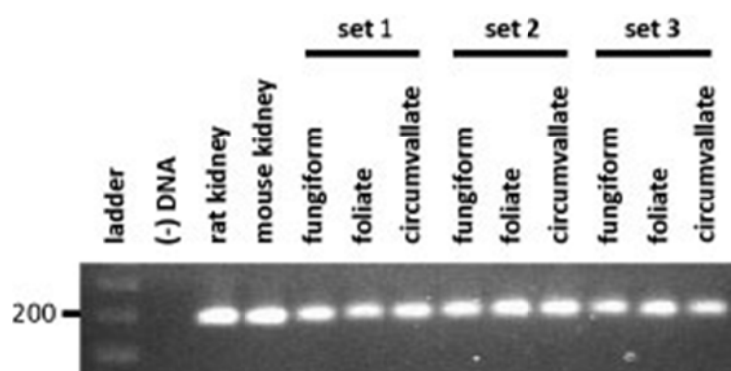


Figure 2.6 RT-PCR showing expression of TRPM4 channels in mouse taste cells. Ethidium bromide-stained gel of PCR products showing TRPM4 was expressed in three sets of pooled mouse taste buds from the fungiform, foliate and circumvallate papillae. Positive controls for TRPM4 using rat kidney cDNA and mouse kidney cDNA are shown. The negative control lane [(-) DNA] represents the omission of cDNA from the PCR reaction.

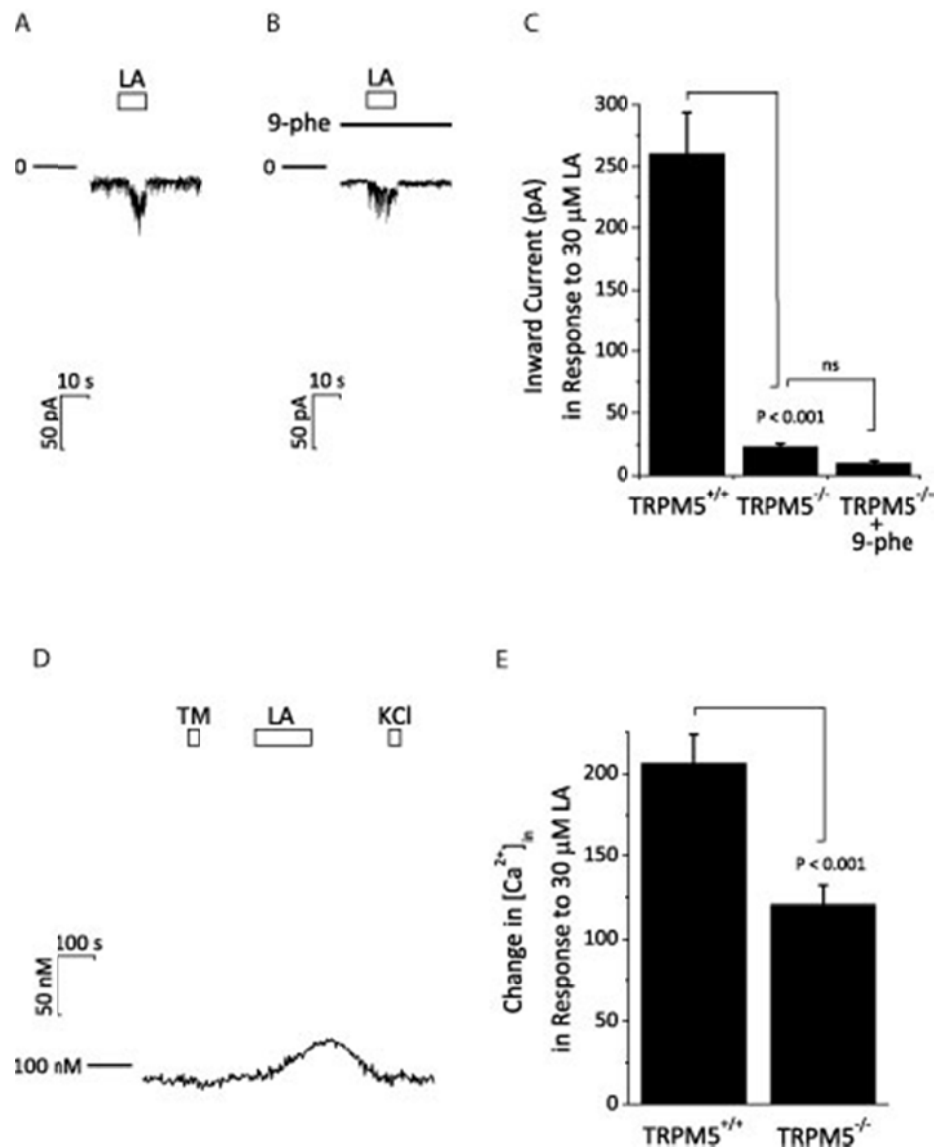


Figure 2.7 LA-induced currents were carried by TRPM5 channels. LA was applied at a final concentration of 30  $\mu$ M for (A)-(D). LA-induced inward current in taste cells isolated from TRPM5<sup>-/-</sup> mice in Tyrode's (A) and in presence of the TRPM4 antagonist 9-phenanthrol (100  $\mu$ M) (B). (C) Mean responses (peak inward current)  $\pm$  S.E.M. to LA in Tyrode's (n = 5) in taste cells from TRPM5<sup>+/+</sup> mice, and to LA in Tyrode's with (n = 10) and without (n = 12) 9-phenanthrol (100  $\mu$ M) treatment in taste cells from TRPM5<sup>-/-</sup> mice. (D) LA-induced intracellular  $Ca^{2+}$  rise in single type II taste cells isolated from TRPM5<sup>-/-</sup> mice. TM, tastant mixture. (E) Mean responses (change in intracellular  $Ca^{2+}$  concentration)  $\pm$  S.E.M. to LA in single Type II taste cells isolated from TRPM5<sup>+/+</sup> (n = 49) and TRPM5<sup>-/-</sup> mice (n = 67).

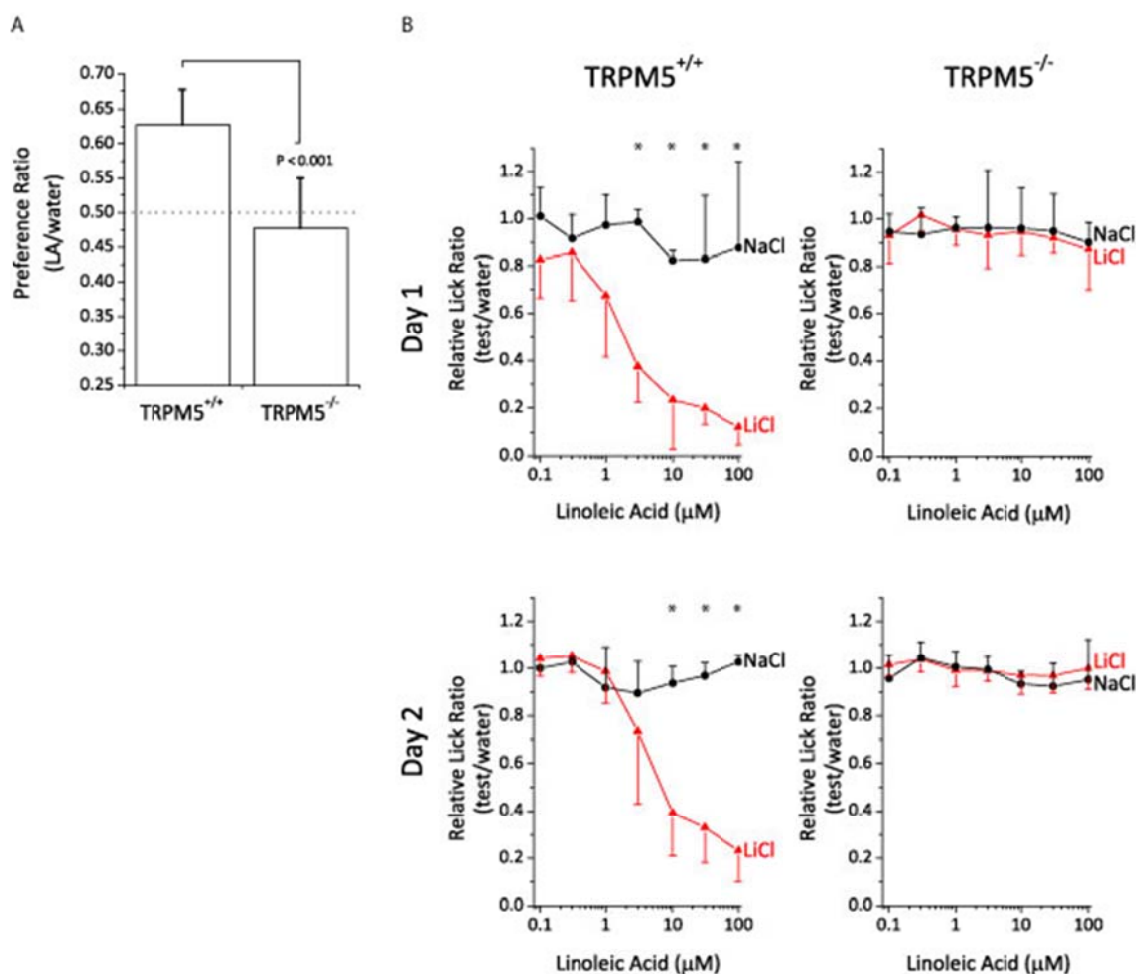


Figure 2.8 Mice lacking TRPM5 channels exhibit no preference for and show reduced sensitivity to LA. (A) Preference ratio for LA compared to vehicle in TRPM5<sup>+/+</sup> and TRPM5<sup>-/-</sup> mice during two-bottle 48-hour LA vs. vehicle tests. LA was applied at a final concentration of 30 μM. TRPM5<sup>-/-</sup> mice (n = 8) were indifferent to a LA emulsion, whereas TRPM5<sup>+/+</sup> (wild-type) mice (n = 8) preferred LA to vehicle (water). (B) Mean lick ratios  $\pm$  S.D. for LA in TRPM5<sup>+/+</sup> and TRPM5<sup>-/-</sup> male mice on day 1 and day 2 after CTA. TRPM5<sup>+/+</sup> LiCl, n = 7; TRPM5<sup>+/+</sup> NaCl, n = 3; TRPM5<sup>-/-</sup> LiCl, n = 9; TRPM5<sup>-/-</sup> NaCl, n = 5. Asterisks indicate significant differences between LiCl-injected (triangle, solid red line) and NaCl-injected (dot, solid black line) groups ( $p < 0.005$ ).

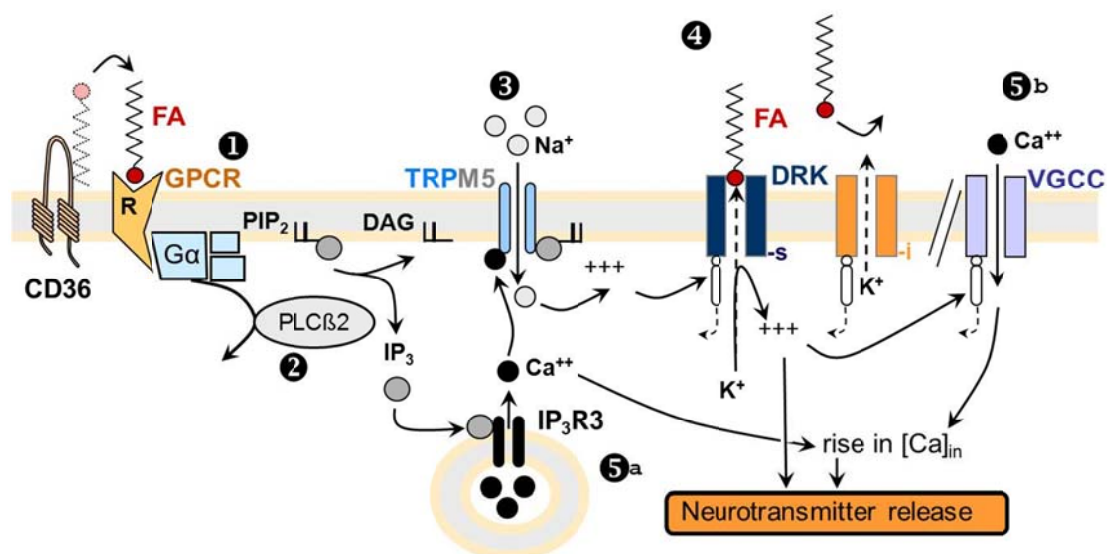


Figure 2.9 Hypothesized putative fat transduction pathway in taste cells. Fatty acids bind to GPCRs and activate G proteins that stimulate PLCβ2, which in turn catalyzes the reaction to generate DAG and IP<sub>3</sub>. IP<sub>3</sub> binds to its receptors on the endoplasmic reticulum and releases calcium. The direct binding of calcium ions or the depletion of intracellular calcium stores activates TRPM5 channels whose activation allows the influx of sodium and further depolarizes the cell. This depolarization opens DRK channels. Inhibition of fatty acid-sensitive DRK channels by fatty acids further reinforces and prolongs the depolarization. This depolarization opens VGCCs leading to a calcium rise in the cytosol. The combined action of depolarization and elevated cytoplasmic calcium level eventually causes the release of neurotransmitters. FA: fatty acid; GPCR: G protein-coupled receptor; TRPM5: transient receptor potential channel type M5; DRK: delayed rectifying potassium channel; VGCC: voltage-gated calcium channel.

CHAPTER 3

FATTY ACIDS ELICIT RESPONSES IN BOTH

TYPE II AND A SUBSET OF TYPE III

MOUSE TASTE CELLS

Abstract

Recently, several fatty acid-activated receptors have been identified that may play a role in fatty acid transduction, and their expression in taste tissues has been verified (1-8), all of which support the idea that fat may reflect an additional primary taste quality. However, which type of taste cells respond to fatty acids remains unclear. Here I show that both type II and type III taste cells express fatty acid-activated receptors. Fatty acids elicit a robust intracellular calcium rise primarily in type II taste cells and a subset of type III taste cells. However, a significant subset of type II taste cells respond to high KCl which has been broadly used as the indicator for type III taste cells as well, suggesting the expression of voltage-gated calcium channels (VGCCs) in these cells. Furthermore, I show that a subset of type II taste cells exhibit VGCC current, verifying the expression of VGCCs in these cells. These results question the utility of being able to use high KCl solution to identify unequivocally type III taste cells within the taste buds, and suggest that the current model of cell typing and cell-to-cell communication within the taste bud may need to be revisited.



## Introduction

Taste buds, which are distributed across different papillae of the tongue, are collections of approximately 50-150 taste cells specialized for the detection of different taste stimuli in the aqueous saliva through a small taste pore. It is now widely accepted that there are three types of mature taste cells within the taste buds termed type I, II, and III taste cells (9). Type I taste cells are usually considered to be the supporting cells within taste bud. Several studies have shown that they may contribute to modulating the extracellular environment within the taste bud (9-10) or directly involved in the salty taste (11-12). Type II taste cells (taste receptor cells) express G protein-coupled receptors (GPCRs) and signaling components for sweet, bitter, and umami compounds. The binding of taste stimuli to the apical GPCRs on type II taste cells initiates a signaling pathway involving the activation of G protein and the  $\beta 2$  isoform of phospholipase C (PLC $\beta 2$ ), production of inositol 1,4,5-triphosphate (IP $_3$ ), release of calcium ions from intracellular stores, and activation of transient receptor potential channel type M5 (TRPM5) whose opening provides the depolarization needed to initiate the action potentials. The intracellular calcium rise and the action potentials both trigger the neurotransmitter ATP release from type II cells onto neighboring type III cells or gustatory afferent nerve fibers (9, 10, 13-19). Type III taste cells (presynaptic cells) receive and integrate signals from type II taste cells, and transfer taste information to the central nervous system (CNS) by forming synapses onto primary afferent nerves. Recent studies show that type III cells also directly respond to sour taste stimuli and carbonated solutions and thus very likely to be the cells responsible for signaling these sensations

(20-23). Recently, several solutions have been widely used as “indicator” solutions to determine taste cell types. Responses to a taste mixture which contains sweet, bitter, and umami compounds were used to identify type II taste cells, and responses to high KCl solutions were used to determine type III taste cells (23-25).

For many years, it was considered that fat and the components contained therein were perceived by their textural properties (26, 27). However, there have been a number of studies that demonstrate the ability of components in fats, specifically free fatty acids, to activate taste cells, suggesting that fat may represent another taste quality (1, 2). Fatty acid-responsive proteins and receptors have been found to be present on the membrane of taste cells (1-8). Recently, I have demonstrated that fatty acids can induce an increase in intracellular calcium concentration and depolarize taste cells. Using a multidisciplinary approach that ranges from the molecular level to the study of taste-guided behaviors in normal and transgenic animal models, I also show that fatty acids activate taste cells through the activation of TRPM5 channels (see Chapter 2). However, which type of taste cells respond to fatty acids still remains unclear.

In the present study I used the “indicator” solutions and followed the procedure published to determine the subtypes of taste cells that respond to fatty acids. Here I show that fatty acids are able to elicit intracellular calcium rise in both type II and a subset of type III taste cells, which have been verified in transgenic mice expressing enhanced green fluorescent protein (GFP) under control of the PLC $\beta$ 2 (GFP-PLC $\beta$ 2) (28) or GAD67 (GFP-GAD67) (23, 29) promoter. However, fatty acids can only activate a non-selective monovalent cation-based inward current, very likely through TRPM5 channels,

in type II but not type III taste cells. More surprisingly, a small subset of type II cells also respond to high KCl, suggesting the expression of VGCCs in these cells, which conflict with previous studies that type II cells lack VGCCs. Further research using GFP-PLC $\beta$ 2 mice show that a subset of type II taste cells exhibit functional VGCC current, verifying the expression of VGCCs in these cells. Fatty acids used in the study vary in their chain length and degree of unsaturation, which may provide a broader view of the “taste of fat”.

## Materials and Methods

### **Animals**

The GFP-PLC $\beta$ 2 and GFP-GAD67 strains have been described in detail previously (23, 28, 29). All experiments were performed on adult (2-6 month) male C57BL/6J, GFP-PLC $\beta$ 2 or GFP-GAD67 mice that were maintained on a 12-h:12-h day/night cycle with normal mouse chow and water provided ad libitum. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Utah State University and were performed in accordance with American Veterinary Medical Association guidelines.

### **Taste Cell Isolation**

Details of single taste cell isolation have been described in detail previously (1,2). Briefly, mice were euthanized by exposure to CO<sub>2</sub> in a closed chamber followed by cervical dislocation and their tongues were removed and placed in ice cold Tyrode's solution. Tongues were injected beneath the lingual epithelium with normal physiological saline (Tyrode's) solution to which was added 1 mg/ml collagenase I (Roche Applied

Science, Indianapolis, IN), 2.45 mg/ml dispase II (Roche Applied Science, Indianapolis, IN), and 1 mg/ml trypsin inhibitor (type I-S; Sigma-Aldrich, St Louis, MO). The amount of the enzyme cocktail solution injected to the anterior portion containing the fungiform papillae, and the area surrounding the two foliate and the circumvallate papillae was 0.2, 0.2, and 0.3 ml, respectively. The tongue was placed in Tyrode's, bubbled with O<sub>2</sub> and incubated for 45 min at room temperature. Following the incubation, the lingual epithelium was removed from the underlying muscle and pinned out in a dish containing a calcium-magnesium free Tyrode's solution and incubated for approximately 10 min. The epithelium was washed with Tyrode's several times and incubated in the same enzyme solution for approximately 7 min. The epithelium was then washed with Tyrode's and incubated in calcium-magnesium free Tyrode's for 5 min. Individual taste cells were moved from the epithelium by gentle suction from a fire-polished pipette (~100 µm pore) and plated immediately into a recording chamber containing Tyrode's for patch clamp recording experiments, or onto a coverslip coated with Cell-Tak Cell and Tissue Adhesive (BD Biosciences, San Jose, CA) for calcium imaging. GFP-labeled cells were identified and collected into a 0.5 ml microfuge tube on ice for the quantitative real time PCR (qPCR) assays.

### **Solutions**

Standard extracellular saline solution (Tyrode's) contained (in mM): 140 NaCl, 5 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. Barium saline (Barium Tyrode's) contained (in mM): 103 BaCl<sub>2</sub>, 10 TEA Bromide, 0.005 TTX, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, and 10

Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. Calcium-magnesium free saline (calcium-magnesium free Tyrode's) contained (in mM): 140 NaCl, 5 KCl, 2 BAPTA, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. A cesium-based intracellular solution was used for recording TRPM5 or VGCC currents and contained (in mM): 140 Cs acetate, 1 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10 HEPES, 11 EGTA, 3 ATP, and 0.5 GTP; pH 7.2 adjusted with TrisOH; 310 mOsm. This cesium-based intracellular solution helped to eliminate most of the voltage-activated outward potassium current. Fatty acids including LA, caproic acid (CA), myristic acid (MA), oleic acid (OA), and arachidonic acid (AA) were made as stock solutions (25 mg/ml) in EtOH, evacuated with N<sub>2</sub> and stored at -20 °C for up to 2 wk until they were diluted for use immediately prior to the experiment. Hanks' buffered salt solution with HEPES buffer and Na pyruvate with fetal bovine serum (HHP with 2% FBS) contained: 98% 1× HBSS, 1% HEPES, 1% Na pyruvate, and 2% heat-inactivated fetal bovine serum; stored at 4°C for up to 1 week. Tastant mixture contained: 20 mM saccharin, 100 μM SC45647, 10 mM denatonium benzoate, 100 μM cycloheximide, and 5 mM monosodium glutamate. 100 mM KCl solution contained (in mM): 45 NaCl, 100 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with KOH; 310 mOsm.

### **Patch Clamp Recording**

Recordings were made from individual taste cells by using the whole-cell patch clamp configuration (1, 2). Taste cell types can be identified using the GFP label under the microscope. LA-induced currents and voltage-gated currents were measured with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA). Patch pipettes were

pulled on a Sutter P-97 puller (Sutter Instruments, Novato CA) and subsequently fire polished on a microforge (model MF-9; Narishige, East Meadow NY) to a resistance between 5 and 10 M $\Omega$  when filled with intracellular solution. Series resistance and cell capacitance were compensated optimally before the recording. Commands were delivered and data were recorded with pCLAMP software (versions 10, Molecular Devices, Sunnyvale, CA) interfaced to an AxoPatch 200 B amplifier with a Digidata 1322 A analog-to-digital board (Axon Instruments, Union City, CA). Data were collected at 5 kHz and filtered on-line at 1 kHz. LA-induced TRPM5 currents in taste cells were recorded using the voltage clamp mode. Typical inward currents were recorded at a holding potential of -100 mV. Linoleic acid (LA) was applied focally from a pipette positioned near the cell and delivered by a PicoSpritzer III (Parker Hannifin Corp, Cleveland, OH) controlled by the data acquisition and analysis software. Voltage-gated currents were recorded using the voltage clamp mode. Holding potential was -100 mV, and the membrane was stepped from -100 mV to +40 mV with 10 mV step to elicit the voltage-gated currents. Note here that as soon as whole-cell configuration was established, extracellular solution was switched to Barium Tyrode's within in 20 s and voltage-gated currents (i.e. VGCC currents) were recorded. Then extracellular solution was switched back to Tyrode's and voltage-gated currents were recorded again to make sure the cell was in good quality.

### **Calcium Imaging**

Single taste cells were loaded with Fura-2AM (5  $\mu$ M; Molecular Probes, Eugene, OR) for 1 h in HHP with 2% FBS at room temperature in the dark. The coverslips were

then mounted onto an imaging chamber (RC-25F, Warner Instruments, Hamden, CT), placed on an inverted microscope (Nikon, Eclipse TE2000-S, Japan) and perfused continuously with normal saline (Tyrode's) solution. Cells were illuminated with a 100-watt xenon lamp and excitation wavelengths (340/380 nm) were delivered by a monochromator (Bentham FSM150, Intracellular Imaging Inc., Cincinnati, OH) at a rate of 20 ratios per minute. GFP-labeled taste cells were identified using excitation wavelength at 470 nm. Fluorescence was measured by a CCD camera (DVC-340M, DVC Company, Austin, TX) coupled to a microscope and controlled by imaging software (Incyt Im2TM, Intracellular Imaging). The ratio of fluorescence (340 nm/380 nm) was directly converted to calcium concentrations using a standard curve generated for the imaging system using Fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR). Fatty acids and other compounds were applied into the extracellular solution through a bath perfusion system at a flow rate of approximately 4 ml/min which permitted complete exchange of the extracellular solution in less than 20 s. I recorded the resting calcium baseline for at least 30 s before each stimulus. To be included in subsequent analyses, calcium responses had to meet two criteria. First, the responses could be triggered repetitively in the same cell by the same stimulus. Second, the peak response (magnitude) was at least ten times the variance of baseline (i.e. prestimulus) fluctuation. To look at the responses of taste cells to more than one type of Fatty acids, fatty acids were tested in random order. TM and KCl were tested in random order as well. No differences in the responses were noted using different stimulus presentation order.

## qPCR

For qPCR, the same type of taste cells, identified by the GFP label, were collected under the microscope and stored in TRI Reagent (MRC, Cincinnati, OH) for immediate extraction of RNA. Extraction was done according to manufacturer's protocol. cDNA was synthesized using the Message*BOOSTER*<sup>TM</sup> cDNA Synthesis Kit for qPCR (EPICENTRE Biotechnologies, Madison, WI). Commercially available TaqMan® Gene Expression Assays (ABI, Carlsbad, CA) were then used to detect the expression of CD36, GPR40, GPR120, TRPM5 and nucleoside triphosphate diphosphohydrolase (NTPDase).

## Results

### **LA Elicits an Intracellular Calcium Rise in Both Type II and a Subset of Type III Taste Cells.**

To explore which type of taste cells responds to fatty acids, I first performed functional calcium imaging using LA as the sample FA. I loaded single taste cells isolated from both fungiform and circumvallate papillae with the ratiometric fluorescent dye Fura-2AM and measured the LA-induced intracellular calcium change. The taste mixture and high KCl solutions were used to determine the taste cell types. My data showed that LA elicited a robust and reversible increase in intracellular calcium in both type II and a subset of type III taste cells (Figure 3.1A, B). To verify my data, I performed similar experiments in the GFP-PLC $\beta$ 2 and GFP-GAD67 labeled taste cells, considered to be representative of type II and type III taste cells, respectively. The LA-induced intracellular calcium rise was observed in both GFP-PLC $\beta$ 2 and GFP-GAD67



labeled taste cells (Fig 3.1C, D), verifying that both type II and a subset of type III taste cells respond to LA.

### **LA Activates a Monovalent Cation-Selective Inward Current in Type II Taste Cells Only.**

My previous studies have shown that rapid and focal application of LA caused a fast and reversible monovalent cation-selective inward current primarily through TRPM5 channels (see Chapter 2). Since TRPM5 channels have been proven to be expressed in type II taste cells only (see Chapter 2), here I tested in which type of taste cells I can record this LA-induced inward current. Using whole-cell voltage clamp recording, I found that LA initiated the inward currents in GFP-PLC $\beta$ 2 labeled taste cells within less than 1 sec (Figure 3.2A), consistent with a role of TRPM5 channels in fatty acid transduction in type II taste cells. On the contrary, no inward current was observed upon the application of LA in the GFP-GAD67 labeled taste cells tested (Figure 3.2B), suggesting that TRPM5 channels are not involved in the fatty acid transduction pathway in type III taste cells. Thus, although both type II and a subset of type III taste cells respond to fatty acids, there might be different transduction mechanisms triggered by fatty acids in these two cell types.

### **Both Type II and Type III Taste Cells Express FA-Responsive Proteins.**

Because the calcium imaging and patch clamp recording experiments suggested that fatty acids activate type II and a subset of type III taste cells very possibly through different transduction pathways, I hypothesized that type II and III taste cells express

different FA-responsive proteins. To test this hypothesis, I pooled 20~40 GFP-PLC $\beta$ 2 or GFP-GAD67 labeled taste cells and processed the extracted RNA for qPCR for CD36 (3, 4), the fatty acid binding protein, and GPR40 and GPR120 which are known receptors for long-chain free fatty acids (5-8). As a control, I also checked the expression of TRPM5 and NTPDase (9), the molecular marker for type I taste cells, to make sure the pooled cells were not contaminated by non-GFP labeled cell. The results (Table 3.1) showed that GPR120 and GPR40 are expressed in both GFP-PLC $\beta$ 2 and GFP-GAD67 labeled taste cells, consistent with the findings that both type II and a subset of type III taste cells responded to LA, the long chain free FA. CD36 was detected in GFP-PLC $\beta$ 2 labeled taste cells only, which may underlie the different transduction mechanisms used in type II and type III taste cells. TRPM5 was detected in GFP-PLC $\beta$ 2 but not GFP-GAD67 labeled taste cells, and NTPDase was detected in neither GFP-PLC $\beta$ 2 nor GFP-GAD67 labeled taste cells, consistent with the interpretation that the harvested cells were likely not contaminated with other taste cell types.

#### **A Subset of Type II Taste Cells Responds to High KCl Solutions.**

VGCCs are usually associated with conventional synapses. In neurons, these channels are responsible for the depolarization-induced calcium influx required for vesicular release. Previous studies showed that depolarization with high KCl solutions did not cause an increase in intracellular calcium concentration in type II taste cells responsible for sweet, bitter and umami taste transduction, suggesting the lack of VGCCs in these cells (30, 31). On the contrary, type III taste cells, which form conventional

synapses onto the gustatory afferent nerves, express VGCCs and exhibit depolarization-dependent calcium transients typically associated with neurotransmitter release (32). Surprisingly, I found in calcium imaging experiments that a significant subset of type II taste cells, indicated by their responses to the taste mixture, in both circumvallate and fungiform papillae, also responded to high KCl solutions (Figure 3.3A, D), suggesting the expression of VGCCs in these cells. The high KCl-induced intracellular calcium rise in these cells was fast (within less than 1 sec) and reversible (Figure 3.3A). The proportions of the type II taste cells which also responded to high KCl solutions were especially remarkable in circumvallate papillae (109 out of 161 taste mixture-responsive type II taste cells) compared to those in fungiform papillae (34 out of 188 taste mixture-responsive type II taste cells) (Figure 3.3D). To explore if VGCCs are expressed in a subset of type II taste cells, GFP-PLC $\beta$ 2 mice were used in calcium imaging experiments. High KCl solutions elicited robust and reversible intracellular calcium rise in over 75% (55 out of 73) of GFP-PLC $\beta$ 2 labeled taste cells isolated from circumvallate papillae (Figure 3.3B, E), the proportion of which was consistent with that from the wild type (WT) animals (Figure 3.3D). Compared with the findings in GFP-PLC $\beta$ 2 labeled taste cells, over 90% (57 out of 63) of GFP-GAD67 labeled taste cells responded to high KCl solution. However, none of them responded to the taste mixture (Figure 3.3C, F). Based on these findings, it seems that the taste mixture is still reliable in identifying type II taste cells. However, high KCl solutions may not be able to identify type III taste cells unequivocally.

**A Subset of Type II Taste Cells Exhibits VGCC Currents.**

Since the calcium imaging experiments suggested that a subset of type II taste cells express VGCCs, I then used whole-cell voltage clamp recording of GFP-PLC $\beta$ 2 labeled taste cells as an additional assay for the presence of VGCCs. After the whole cell configuration was established, I quickly (within 20 s) switched the extracellular solution from the normal Tyrode's solution to a Barium Tyrode's solution which contained BaCl<sub>2</sub>, TTX, and TEA (30, 31), and applied 10 mV depolarizing voltage steps from a holding potential of -100 mV. Eight out of 33 GFP-PLC $\beta$ 2 labeled taste cells exhibited large, slowly inactivating inward currents (Figure 3.4A), confirming that a subset of type II taste cells express functional VGCCs. The current-voltage profile of this current suggested that it was elicited by high-voltage activated calcium channels, likely L-type VGCCs. To confirm the taste cells were of good quality and exhibit normal electrophysiological properties, after recording in the Barium Tyrode's, I usually switched the extracellular solution back to normal Tyrode's solution and tested if the cell exhibit normal voltage-gated sodium and potassium currents. After switching back to normal Tyrode's solution, all GFP-PLC $\beta$ 2 labeled taste cells tested (n = 33) exhibited voltage-gated sodium and potassium currents in response to 10 mV depolarizing steps from a holding potential of -100 mV (Figure 3.4B). As a control for the methodology, I also tested several GFP-GAD67 labeled taste cells which were known for the expression of functional VGCCs. All of them displayed a large, slowly inactivating inward current in the Barium Tyrode's and large voltage-gated sodium and potassium currents in normal

Tyrode's (data not shown). No significant differences were found between the VGCC currents recorded in GFP-PLC $\beta$ 2 and GFP-GAD67 labeled taste cells.

Surprisingly, VGCC current was relatively large (300~500 pA) when whole cell was first established, but then washed out fairly quickly (within 3 min) after whole cell configuration (Fig 3.4C, D). This may explain why VGCC current was rarely seen in type II taste cells electrophysiologically in the past studies in which voltage-gated currents were usually recorded in normal Tyrode's first (30). Our findings strongly suggest that VGCCs are expressed in subpopulations of type II taste cells, and question the utility of being able to use high KCl solution to identify unequivocally type III taste cells within the taste bud.

#### **CA, MA, OA, or AA Activates Both Type II and a Subset of Type III Taste Cells.**

For most of the experiments including calcium imaging and electrophysiological experiments, I used LA (C18:2 cis, cis-9,12) as the representative FA. After I found both type II and a subset of type III taste cells responded to LA, I tested more fatty acids that varied in their chain length and degree of unsaturation: CA (C6:0) is a short chain saturated FA; MA (C14:0) is a medium chain saturated FA; OA (C18:1 cis-9) is a long chain mono-unsaturated FA; AA (C20:4 all-cis-5,8,11,14) is a long chain polyunsaturated FA. This would give us a broader picture about how different types of taste cells respond to fatty acids. Calcium imaging showed that all of them elicited an intracellular calcium rise in both type II and a subset of type III taste cells (Figure 3.5). The responses to CA, MA and AA were comparably slower and had a more gradual onset (Figure 3.5A, B, D,

E, F, H), while the responses to OA were fast and immediate (Figure 3.5C, G), which might due to their binding to different FA-responsive receptors.

### **Cell Typing Analysis of LA, CA, MA, OA, and AA**

Because our data suggest that fatty acids varying in chain length and degree of unsaturation can all activate both type II and a subset of type III taste cells, I further analysed my calcium imaging data to look at the cell typing and grouping of the five types of fatty acids tested, which was summarized as Venn diagrams shown in Figure 3.6. The proportions and types of taste cells that responded to each of the fatty acids tested vary from one fatty acid to another, which may be due to the different expression assays of FA-responsive receptors on different taste cells. Furthermore, I tested multiple fatty acids in the same experiment on taste cells. The results showed that one taste cell can respond to more than one type of fatty acids. A subset of taste cells responded to all four types of fatty acids tested including CA, MA, OA, and LA (Figure 3.7A). A K-diagram was made to show the grouping of taste cells that responded to CA, MA, OA, and LA (Figure 3.7B).

## **Discussion**

In 1997, Gilbertson *et al.* provided the first direct evidence that fatty acids elicit a gustatory (i.e., taste) cue in rat taste receptor cells (1), suggesting the presence of a sensory mechanism for fat in taste cells. Since then, several other FA-responsive proteins were identified and found to be expressed in the gustatory system, including CD36 (3, 4),

the fatty acid binding protein, and several GPCRs (5-8). These findings challenged the notion that fats provide solely textual cues in the oral cavity (26, 27). The effects of fatty acids in the gustatory system might be important for understanding how our body recognizes and responds to dietary fat. Our very recent studies have shown that fatty acids can activate taste cells as an independent taste stimuli and initiate intracellular calcium rise and depolarization of the cell. TRPM5 channels play an essential role in this process (see Chapter 2). However, due to the heterogeneity of taste cells, it would be interesting to see which type of taste cells respond to fatty acids.

Using taste cells from both wild type and GFP-PLC $\beta$ 2 and GFP-GAD67 mice, here I show that fatty acids caused a robust and reversible increase in the intracellular calcium concentration in both type II and a subset of type III taste cells (Figure 3.1). However, fatty acids only initiated an inward current, primarily through TRPM5 channels, in type II but not type III taste cells (Figure 3.2), suggesting that the transduction mechanisms in different cell types might be different. Since TRPM5 channels have been suggested to play a critical role in fatty acid transduction and their expression in type II taste cells has been verified (Table 3.1) (15, 33), it is very possible that fatty acids activate type II taste cells through the activation of TRPM5 channels. On the contrary, type III taste cells lack TRPM5 channels. Thus the fatty acid transduction in type III taste cells might be very different from that in type II taste cells.

The differences in the transduction between type II and type III taste cells might be due to the expression of different FA-responsive proteins or receptors on the apical membrane. A recent report suggested that both GPR120 and GPR40 contribute to fatty

acid signaling. Using immunohistochemistry, it is shown that GPR120 is primarily expressed in type II taste cells. 80% of GPR40 is expressed in Type I taste cells, with the remaining 20% expressed in type II and type III taste cells (8). Consistent with this finding, our molecular data using RNA from pooled GFP labeled cells showed that both type II and type III taste cells express GPCRs for long chain free FA, including GPR120 and GPR40 (Table 3.1). However, CD36 was only detected in GFP-PLC $\beta$ 2 (type II) but not GFP-GAD67 (type III) labeled taste cells. Previous studies have shown that taste cells expressing CD36 respond to LA by increasing IP<sub>3</sub> and intracellular calcium concentrations, SRC-kinase phosphorylation, and neurotransmitter release (34). The role of CD36 in fatty acid transduction might be to bring fatty acid molecules to the actual receptors. The expression of CD36 in type II taste cells only may underlie the different transduction pathways between type II and type III taste cells. Calcium imaging followed by single-cell RT-PCR may help unravel this puzzle in the future studies.

Another major finding in this report is that subpopulations of type II taste cells also responded to the high KCl solution (Figure 3.3), suggesting the expression of VGCCs in these cells. To confirm this finding, I have successfully recorded VGCC current in 8 out of 33 GFP-PLC $\beta$ 2 labeled taste cells (Figure 3.4A). These data are consistent with a previous study showing that one subset of Antigen A-immunoreactive type II taste cells exhibited large inward sodium and outward potassium as well as VGCC current (31). The results may also help explain the observation that sweet stimuli can generate large trains of action potentials (35-37). However, Clapp *et al.* reported that no GFP-T1R3 labeled taste cells, the sweet-responsive taste cells, responded to high



potassium depolarization stimulus ( $n = 21$ ) (30). DeFazio *et al.* also reported that high KCl solution never induced intracellular calcium rise in the taste mixture-responsive type II taste cells. Out of 1032 cells from circumvallate papillae, 5% ( $n = 53$ ) responded to KCl depolarization, 3% ( $n = 34$ ) responded to the taste mixture, whereas none generated an intracellular calcium response to both stimuli (32). This discrepancy is possibly due to the differences in how taste cells were isolated, or the composition of the taste mixture in which I also included 5 mM monosodium glutamate. I do not believe this to be the case. In our preliminary experiments, I recorded responses of taste cells to the taste mixture with and without monosodium glutamate, and didn't see statistically significant differences. Thus for all following experiments, I included monosodium glutamate in the taste mixture to include the umami-responsive type II taste cells in the assay. Another concern is the quality of the cells. In our calcium imaging experiments, more than 40% (295 out of 676) of the taste cells from circumvallate papillae responded to either high KCl solution or taste mixture (Figure 3.6A), which is significantly higher than the proportion (8%, 87 out of 1032) showed in the previous report (32). A possible explanation is that the taste cells used in our report were healthier and more intact, with less damage to the receptors and ion channels on the cell membrane which include the sweet, bitter and umami-responsive GPCRs and VGCCs.

However, VGCC current has been rarely recorded in type II taste cells (30). I found in the whole cell voltage clamp recording that the VGCC currents in type II taste cells were washed out very rapidly, within 3 min after the whole cell configuration (Figure 3.4C, D). Thus, if voltage-gated currents were first recorded in normal Tyrode's

as a control, VGCC currents would be hardly seen after switching the extracellular solution to the Barium Tyrode's. This may explain why VGCC currents have been rarely seen electrophysiologically in type II taste cells in earlier studies in which voltage-gated currents were usually recorded in normal Tyrode's first (30).

To look at the cell typing and grouping of taste cells that respond to fatty acids, I also performed calcium imaging experiments with a variety of fatty acids that vary in their chain length and degree of unsaturation, including short chain saturated fatty acid (CA), medium chain saturated fatty acid (MA), long chain monounsaturated fatty acid (OA), and long chain polyunsaturated fatty acid (LA and AA). The results showed that all the fatty acids tested activated both type II and III taste cells (Figure 3.5, 3.6), consistent with the molecular data that some fatty acid-activated proteins were expressed in both type II and III taste cells. I also found that one taste cell can respond to one to several types of fatty acids. A subset of taste cells responded to all four types of fatty acids tested, including CA, MA, OA, and LA (Figure 3.7). The taste selectivity for fat might be determined by the coexpression of different combinations of GPCRs with CD36 and down-stream signaling molecules such as TRPM5 or fatty acid-sensitive potassium channels.

In conclusion, based on the calcium imaging, patch clamp recording and molecular experiments, I found that fatty acids are able to elicit responses in both type II and a subset of type III taste cells. A subset of type II taste cells responded to high KCl depolarization and exhibited VGCC current, which questions the utility of being able to use high KCl solution to identify unequivocally type III taste cells within the taste buds.

While TRPM5 channels are essential for fat perception in type II taste cells, type III taste cells, however, may use a different fat transduction pathway.

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Table 3.1 qPCR showing expression of the putative fatty acid-responsive proteins in GFP-PLC $\beta$ 2 (type II) and GFP-GAD67 (type III) labeled taste cells.

Targets	GFP-PLC $\beta$ 2		GFP-GAD67	
	Fungiform	Circumvallate	Fungiform	Circumvallate
GPR120	+	+	-	+
GPR40	+	-	+	+
CD36	+	+	-	-
TRPM5	+	+	-	-
NTPDase	+	-	-	-



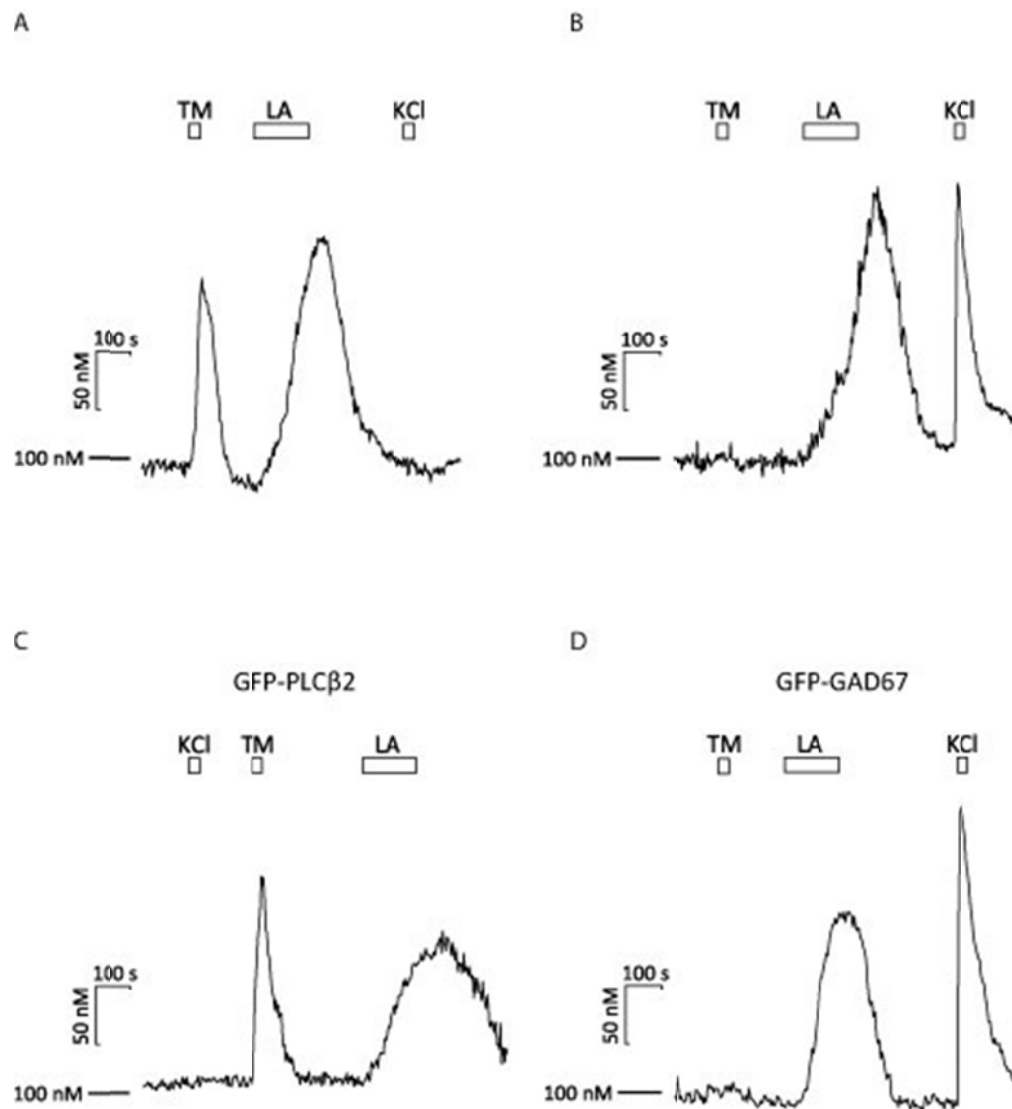


Figure 3.1 LA induced intracellular  $\text{Ca}^{2+}$  rise in both type II and a subset of type III taste cells. LA was applied at a final concentration of 30  $\mu\text{M}$ . (A) Bath application of LA induced a robust intracellular  $\text{Ca}^{2+}$  rise in type II taste cells, indicated by its response to TM. (B) Bath application of LA induced a robust intracellular  $\text{Ca}^{2+}$  rise in type III taste cells, indicated by its response to high KCl. (C) Bath application of LA induced a robust intracellular  $\text{Ca}^{2+}$  rise in GFP-PLC $\beta$ 2 labeled taste cells that also responded to TM. (D) Bath application of LA induced a robust intracellular  $\text{Ca}^{2+}$  rise in GFP-GAD67 labeled taste cells that also responded to high KCl. TM: taste mixture; KCl: high KCl solution.

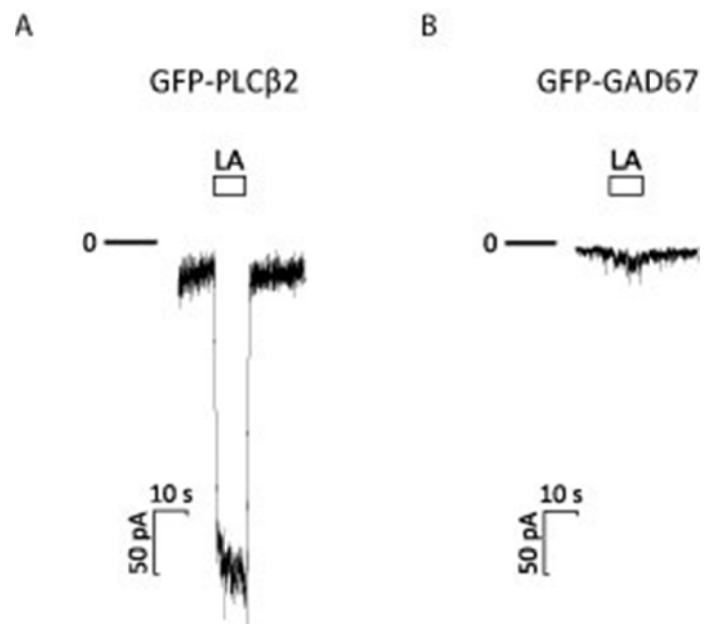
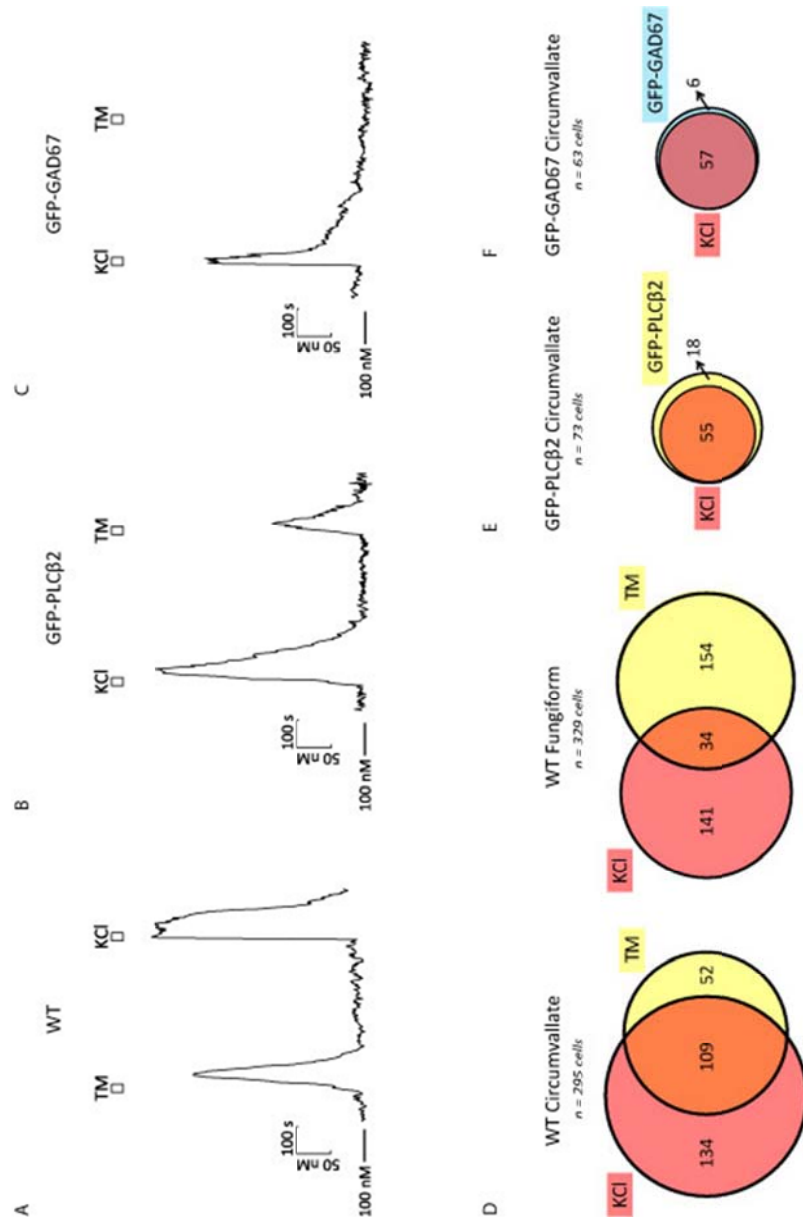


Figure 3.2 Rapid and focal application of 30  $\mu$ M LA elicited a monovalent cation-permeable current in type II but not type III taste cells. LA-induced inward currents (holding potential = -100 mV) in GFP-PLC $\beta$ 2 labeled (type II) taste cells (A) and GFP-GAD67 labeled (type III) taste cells (B).



type II taste cells also responded to high KCl (D); more than 75% (55 out of 73) of GFP-PLC $\beta$ 2 labeled taste cells in circumvallate responded to high KCl (E); more than 90% (57 out of 63) of GFP-GAD67 labeled taste cells in circumvallate responded to high KCl, but none of them responded to TM (F). TM: taste mixture; KCl: high KCl solution.

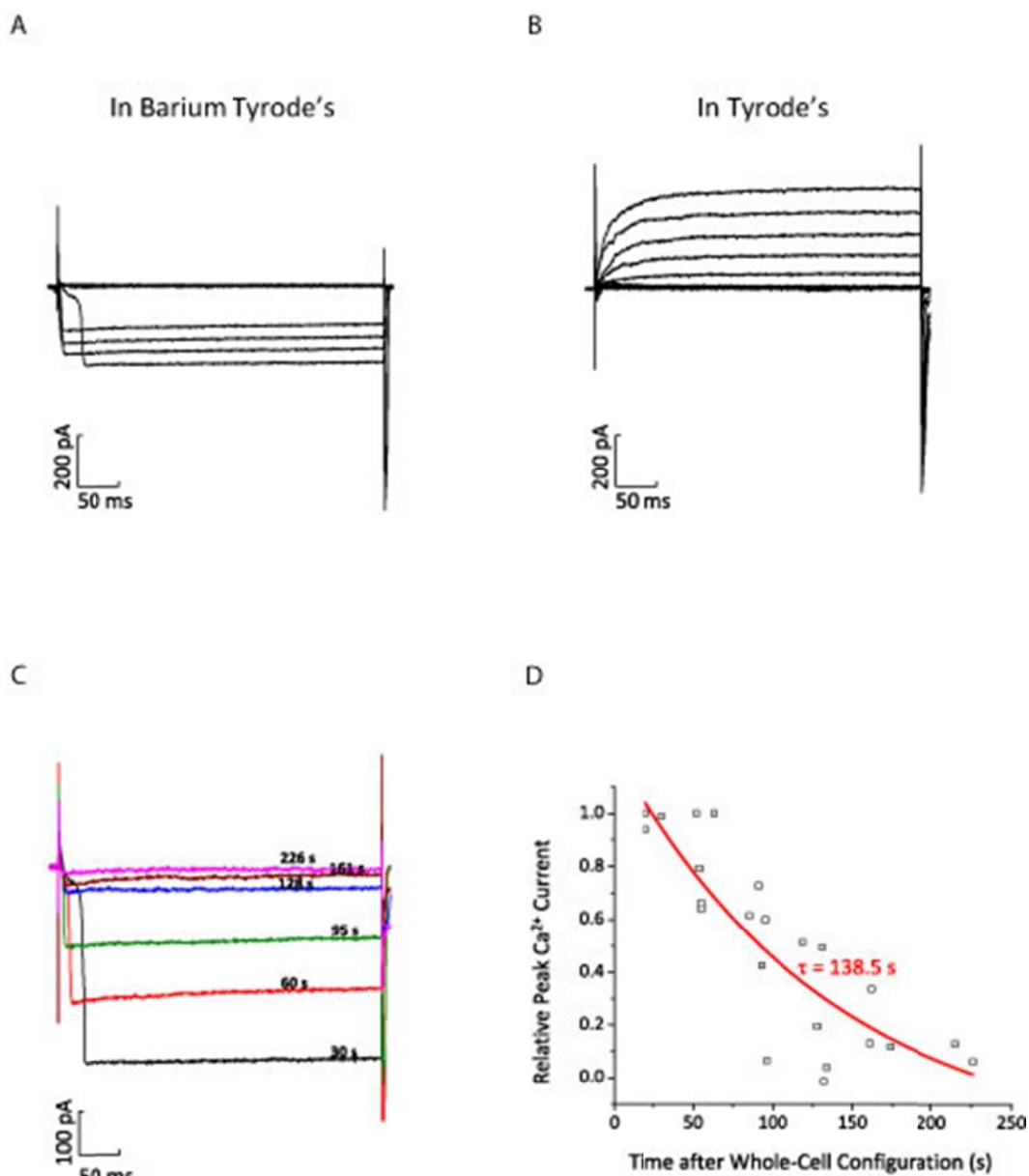


Figure 3.4 A subset of type II taste cells exhibited VGCC currents. Voltage-gated currents recorded in Barium Tyrode's (A) and normal Tyrode's (B). Holding potential was -100 mV, and the membrane was stepped from -100 to +40 mV to elicit voltage-gated currents. (C) A sample cell showing VGCC currents (in Barium Tyrode's) at +10 mV at various times after reaching whole-cell configuration. (D) Time course of the washout of VGCC current.

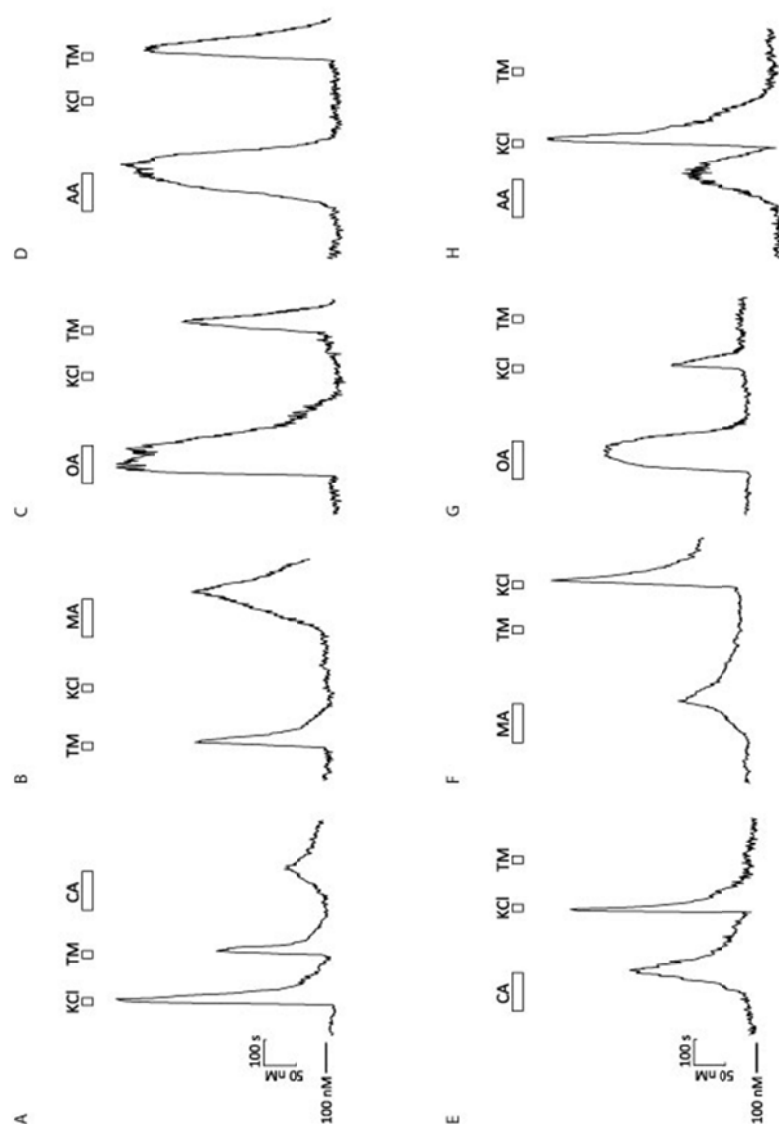


Figure 1. EPR spectra of the paramagnetic complex in the presence of various ligands.

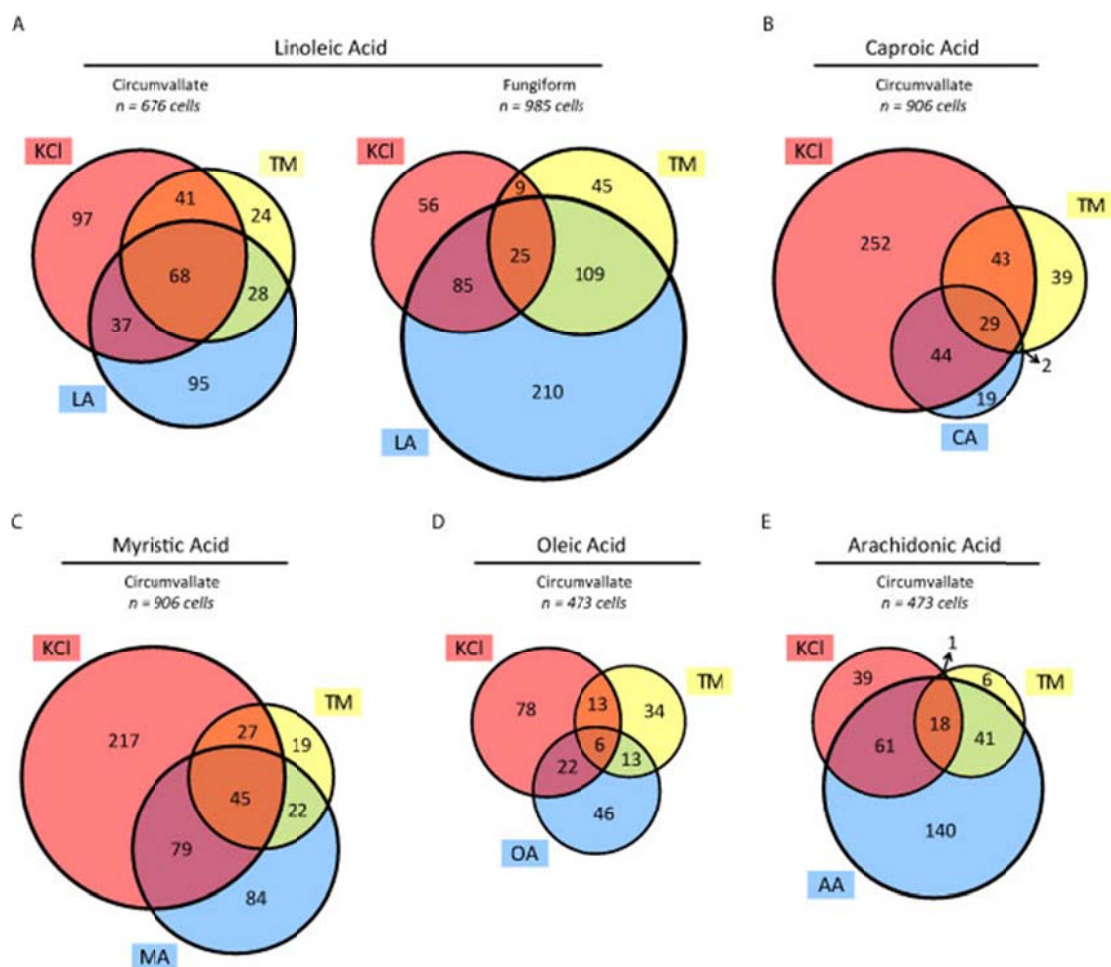


Figure 3.6 Cell typing analysis of LA, CA, OA, MA, and AA. Venn diagrams showing the cell typing of LA in circumvallate and fungiform taste cells (A), CA (B), MA (C), OA (D), and AA (E) in circumvallate taste cells.

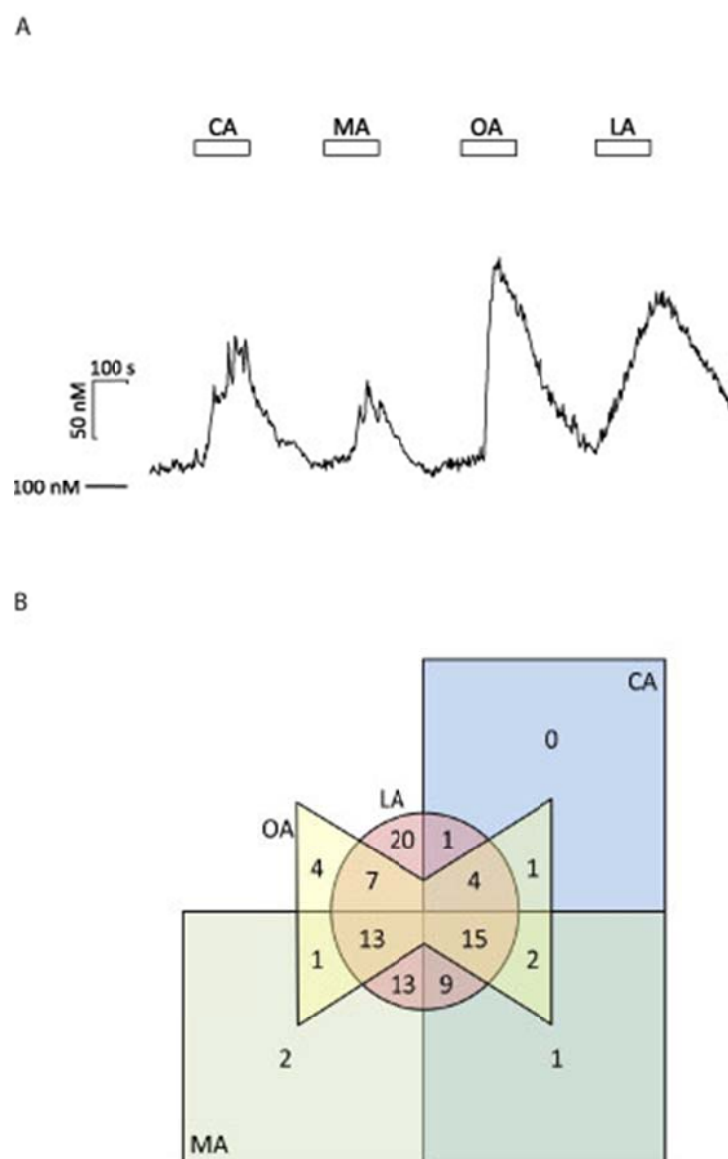


Figure 3.7 Grouping of taste cells that responded to CA, MA, OA, and LA. (A) A subset of taste cells responded to all the fatty acids tested. (B) K-diagram showing the grouping of taste cells that responded to CA, MA, OA, and LA.

## CHAPTER 4

### SUMMARY AND FUTURE DIRECTIONS

Earlier studies in our laboratory suggested that fatty acids can work as a primary taste quality and activate taste cells through the inhibition of DRK channels (1) using the open channel blocker mechanism (2-5). However, only a small proportion (approximately 5%) of DRK channels would be open at the resting membrane potential of taste cells (6), suggesting that there are additional receptors and signaling pathway upstream of the DRK channels that are activated by fatty acid and provide the depolarization necessary to open DRK channels. Thus the primary goal of this dissertation is to explore the fatty acid transduction pathway in mouse taste cells.

To achieve this goal, I first used cell-based assays including patch clamp recording and functional calcium imaging to explore if fat, specifically fatty acids, can activate taste cells as a primary taste quality or not. My results showed that linoleic acids depolarized taste cells significantly (Figure 2.1) and induced a robust intracellular calcium rise (Figure 2.2). Interestingly, despite that numerous research has been done to explore the effects of fatty acids on taste cells, my data were the first to show that fatty acids could induce membrane depolarization and increase intracellular calcium concentration in taste cells. Since the results confirmed my initial hypothesis that fatty acids themselves can activate taste cells as an additional primary taste quality, the next step would be to explore what underlies this depolarization and intracellular calcium rise. By removing sodium or calcium ions from the extracellular solution, or depleting intracellular calcium stores using pharmacological approaches, it is shown that the fatty



acid-induced intracellular calcium rise was a combined effect of a release of calcium ions from intracellular calcium stores and an influx of calcium ions from extracellular environment (Figure 2.1). At the same time, whole cell patch clamp recording experiments showed that the fatty acid-induced depolarization was dependent on extracellular sodium ions but not on extracellular calcium ions, suggesting that the depolarization is downstream of the sodium influx, but upstream of the calcium influx (Figure 2.2).

Using carefully designed voltage-clamp recording, I also showed that rapid and focal application of linoleic acid caused a fast inward current in individual taste cells. And this linoleic acid-induced inward current was significantly reduced when extracellular sodium ions were removed. The following ion substitution experiments showed that the linoleic acid-induced inward currents were carried by monovalent cations (Figure 2.3).

Recently several fatty acid-responsive proteins including the fatty acid-binding protein CD36 (7-10), fatty acid-sensitive DRK channels (1, 11), and several GPCRs (12-17) have been identified. Using RNA isolated from taste bud cells, I found that all of these putative fatty acid-responsive proteins were expressed in taste cells (Table 2.2). Patch clamp recording experiments showed that when G protein or PLC were blocked by GDP- $\beta$ -S or U73122, respectively, the linoleic acid-induced inward current and depolarization were significantly reduced, suggesting that the G protein-PLC pathway is involved in fatty acid transduction (Figure 2.4). This was the first evidence suggesting

that the fatty acid-activated GPCRs may be involved in the fatty acid perception in the gustatory system.

As stated in Chapter 1, the calcium-activated, monovalent cation-selective channel TRPM5 has been shown to play an essential role in the transduction of sweet, bitter and umami tastes (18, 19). Since I have shown that the linoleic acid-induced inward current was monovalent cation-selective and downstream of PLC activation, I hypothesized that TRPM5 channels may be involved in this process. Subsequent experiments showed that the linoleic acid-induced inward currents and intracellular calcium rise were greatly inhibited by the TRPM5 blocker, TPPO (Figure 2.5), and were virtually abolished in taste cells from mice lacking TRPM5 (Figure 2.7). Moreover, TRPM5 null mice lost the preference for and sensitivity to fatty acids, confirming that TRPM5 channels play an essential role in fatty acid transduction (Figure 2.8).

As many details of the fatty acid transduction pathway were studied, a new question emerged: which type of taste cells respond to fatty acid? This became the second major goal of this dissertation research. To identify the subtype of FA-responsive taste cells, I followed a published protocol and used the responses to high KCl as an indicator of type III taste cells and responses to a tastant mixture (sweet, bitter, and umami) to identify type II taste cells (20-22). Fatty acids were able to elicit an intracellular calcium rise in cells that correspond to Type II and a subset of type III taste cells, which have been verified in transgenic mice expressing enhanced green fluorescent protein (GFP) under control of the PLC $\beta$ 2 (GFP-PLC $\beta$ 2) or GAD67 (GFP-GAD67) promoters (Figure 3.1). Surprisingly, our data showed that a small subset of type II cells, identified by their

response to the tastant mixture, also responded to high KCl (Figure 3.3), suggesting the expression of voltage-gated calcium channel (VGCCs) in these cells. This finding conflicted with previous studies that type II cells lacked VGCCs. To explore whether VGCCs are expressed in a subset of type II cells, GFP-PLC $\beta$ 2 mice were used in both functional calcium imaging and patch clamp recording. Calcium imaging data showed that high KCl elicited a robust calcium rise in over half of GFP-PLC $\beta$ 2 labeled taste cells (Figure 3.3), consistent with the expression of VGCCs. Patch clamp recording showed that VGCC currents were present in 8 out of 33 GFP-PLC $\beta$ 2 labeled taste cells (Figure 3.4). My findings strongly suggest that functional VGCCs are expressed in subpopulations of Type II cells and question the current model of cell signaling within the taste bud as well as the utility of high KCl to identify unequivocally presynaptic cells within the taste bud.

To look at the cell typing and grouping of taste cells that respond to fatty acids, I also performed calcium imaging experiments with a variety of fatty acids that vary in their chain length and degree of unsaturation. The results showed that both type II and type III taste cells can respond to a variety of fatty acids (Figure 3.5, 3.6).

To this end my dissertation has elucidated many details of the fatty acid transduction pathway and obtained some initial information about the cell typing that respond to fatty acid. However, several questions revealed by this research remain open and further studies in great depth are warranted. First, I have provided some evidence that fatty acid-activated GPCRs might be involved in fat perception. However, evidence as to how fatty acids bind to their receptors and activate taste cells is still lacking. Many

interesting questions can be addressed on this topic. Do fatty acids directly bind to GPCRs? Or does their insertion into the plasma membrane change the conformation of the GPCRs and trigger the downstream signaling pathway? Are fatty acids transported across the membrane and initiate a transduction pathway on the cytosolic side? Is CD36 needed to facilitate the binding of fatty acids to the GPCRs? If so, how are they organized on the membrane? Are they accumulated into working units by lipid rafts? Second, my data suggests that both type II and a subset of type III taste cells respond to fatty acids, and that TRPM5 channels play an essential role in the fatty acid perception. However, TRPM5 channels are only expressed in type II taste cells (18, 23, Table 3.1). Thus it is very possible that the transduction pathway I suggest in this study primarily reveals fat perception in type II taste cells. Type III taste cells, however, might respond to fatty acids in a very different way. Clearly more research is needed to elucidate this issue in greater detail. Third, although TRPM5 channels have been proven to play a critical role in the fatty acid perception, the mechanisms of their activation/regulation remain contradictory. Some studies show that TRPM5 channels can be directly activated by intracellular calcium (24-26), or the depletion of calcium from intracellular stores (23). However, other studies showed that TRPM5 is not activated by calcium, IP<sub>3</sub>, or depletion of intracellular calcium stores (18). Since some of the studies were done in cell lines, it remains possible that the heterologously expressed channels do not fully recapitulate the properties of native channels, making the mechanisms even more uncertain. Last but not least, despite my progress in elucidating the fatty acid transduction pathway, this research was performed almost entirely upon linoleic acid as the fatty acid stimulus. Thus it is

possible that other fatty acids utilize very different receptors and pathways to activate taste cells. This possibility is suggested by some preliminary data from calcium imaging experiments that showed that the time courses of the intracellular calcium rise induced by different fatty acids were not the same (Figure 3.5). The cell typing that responded to each of the fatty acids tested varied as well (Figure 3.6). Despite these differences, all of the fatty acids tested initiated significant intracellular calcium changes in both type II and a subset of type III taste cells (Figure 3.5). And each taste cells can respond to a variety of fatty acids that vary in their chain length and degree of unsaturation (Figure 3.7). The universality and individuality suggested by my data provide a clue for how taste system encodes the signal of fat taste. Next I will discuss all these open questions in detail.

#### How Do Fatty Acids Bind to Their Receptors and Activate Taste Cells?

My research has shown that when the activation of G protein or PLC was blocked, the fatty acid-induced depolarization and intracellular calcium rise were significantly reduced (Figure 2.4), suggesting that the G protein-PLC pathway is involved in fatty acid transduction. This, in turn, suggested a role of GPCRs in this process. As discussed in Chapter 1, these fatty acid-responsive GPCRs can be activated by a variety of fatty acids that vary in their chain length and degree of unsaturation, and their expression in taste tissues has been verified (15-17, Table 2.2). However, their direct roles in fatty acid transduction have not been verified until recent studies with transgenic mice that lack these GPCRs. Studies using GPR40 knockout mice showed that GPR40 was necessary

but not sufficient for the fatty acid-stimulated insulin release (27). A very recent study showed that male and female GPR120 knockout and GPR40 knockout mice exhibited a diminished preference for linoleic acid and oleic acid, and diminished taste nerve responses to several fatty acids (17), providing direct evidence that GPR120 and GPR40 mediate the taste of fatty acids. Despite these progresses, direct evidence for the role of other GPRs in fatty acid transduction is still lacking. Further studies using other GPCR knockout animal models in both cell-based and behavioral experiments may help validate their roles.

Another open question is how fatty acids activate their corresponding GPCRs. Here I want to bring in another protein that is involved in fat taste transduction, CD36. CD36 has been shown to transport fatty acids into the cytoplasm of myocytes and adipocytes (28). It is expressed in taste cells, and mice that lacked CD36 showed a significantly diminished preference for linoleic acid (9). CD36-expressing taste cells respond to linoleic acid by increasing intracellular calcium concentration, Src-kinase phosphorylation and releasing neurotransmitters (29). The role of CD36 in fatty acid taste has been highly controversial and still remains unclear. Some believe that CD36 is a fatty acid transporter that helps with the movement of fatty acid from extracellular environment to the cytosol. Others argue that CD36 is to bring fatty acid to the actual receptor and acts as a “facilitator.” To answer this question, a series of cell-based assays and biophysical experiments would be helpful. Patch clamp recording or functional calcium imaging using cell lines expressing GPCRs with and without CD36 may reveal whether CD36 works independently or in combination with GPCRs. Raman spectra using

cell lines expressing GPCRs with CD36 could help answer whether CD36 and GPCRs interact or not. Furthermore, high-quality immunocytochemical experiments may help unravel the possible co-localization of CD36 and GPCRs.

Due to their physicochemical properties, fatty acids themselves can insert into or diffuse across the plasma membrane. Do fatty acids activate taste cells by interacting with the lipid bilayer and in turn change the conformation of GPCRs? Or do they diffuse or get transported by CD36 across the membrane and activate the cell on the cytosolic side? Patch clamp recording with fatty acid included in the intracellular solution may help answer whether fatty acids work on the extracellular or cytosolic side of the membrane. Clearly more research needs to be done in the future to fully understand how fatty acids activate taste cells.

### What Are the Mechanisms Underlying Fat

#### Perception in Type III Taste Cells?

My behavioral data showed that the loss of TRPM5 resulted in a complete loss of preference for linoleic acid and the ability to form a CTA against this fatty acid (Figure 2.8), suggesting that Type II taste cells might be the primary receptor cells for fatty acids. A recent report suggested that GPR120 is primarily expressed in fungiform and circumvallate type II taste cells, while GPR40 is expressed mainly in Type I taste cells from the circumvallate papillae (17). Based on my results, I would argue that GPR120 is more relevant to linoleic acid taste since GPR120 is expressed primarily in Type II cells (TRPM5-expressing), which do not apparently express GPR40.

However, my data in Chapter 3 showed that a subset of type III taste cells also responded to fatty acids (Figure 3.1, 3.5). Since type III taste cells do not express TRPM5 channels (18, 23, Table 3.1), they may respond to fatty acids in a very different way and exhibit other functions when fatty acids are presented. This is reasonable since type II and III taste cells differ in their protein expression (30) and their roles in the taste system are quite different (see Chapter 1). Actually my molecular data has revealed that type II and III taste cells express different groups of fatty acid-responsive proteins (Table 3.1), providing the molecular basis for the different mechanisms type II and III taste cells may use.

As stated above, type II taste cells seem to be critical in fatty acid detection, functioning as the “fat taste receptors.” What are the functions of type III taste cells in fat perception? What are the fatty acid-activated signaling pathways in type III taste cells? Since fatty acids are able to modulate the responses of taste cells to other taste qualities (11, 31-35), it is possible that the functional sites of this modulation are in type III taste cells which receive and integrate information from other taste cell types and broadly tuned to multiple taste qualities (see Chapter 1).

Since CD36 was only detected in GFP-PLC $\beta$ 2 labeled (type II) taste cells, I would further hypothesize that the presence of CD36 and its interaction with fatty acids are necessary for fatty acid detection, i.e. the taste of fat. While in type III cells that do not express CD36, fatty acids trigger a different pathway that primarily functions to modulate other taste qualities. To study the fat perception in type III taste cells, both cell-based assays and immunocytochemical experiments using GFP-GAD67 marked (type III)



taste cells are warranted. Calcium imaging followed by single-cell RT-PCR may help unravel this puzzle in the future studies.

### How Is TRPM5 Activated?

A major finding of this dissertation is that TRPM5 is proved to play an essential role in fatty acid perception. However, the mechanisms of its activation or regulation still remain controversial. Patch clamp recording using excised patches from transfected cells that express TRPM5 showed that TRPM5 channels can be directly activated by intracellular calcium (24). Another study using *Xenopus laevis* oocytes and CHO (Chinese hamster ovary) cells showed that TRPM5 is activated by the depletion of calcium from intracellular stores (23). However, other studies showed that TRPM5 is not activated by calcium, IP<sub>3</sub>, or depletion of intracellular calcium stores (18). Till now there has been no agreement on how TRPM5 is activated. Moreover, a big concern is that most of the studies were done in cell lines transfected with TRPM5. Accordingly it remains possible that the heterologously expressed channels do not fully recapitulate the properties of native channels, making the mechanisms even more uncertain.

To examine the role of calcium and internal stores in the activation of TRPM5 channels in native taste cells, one strategy is to deplete intracellular calcium stores with thapsigargin and to manipulate intracellular calcium by release of caged calcium, caged DAG, caged IP<sub>3</sub>, and treatment with BAPTA. This could be difficult because of the challenges that include how to maintain the good quality of taste cells after incubation in thapsigargin for a certain time (at least 5 min based on my observation). But it should be

possible to show whether TRPM5 is activated by direct binding of intracellular calcium ions, DAG, or IP<sub>3</sub>, or the depletion of intracellular calcium stores.

### How Does the Taste System Encode the Signal of Fat Taste?

Most of the studies in this dissertation were based on the polyunsaturated fatty acid, linoleic acid (C18:2 cis, cis-9,12). The choice of using linoleic acid as the fatty acid stimulus was based on our observations that linoleic acid usually initiated comparably large responses, and its function as essential fatty acid. Thus it is possible that the transduction mechanism is only applicable to a small group of fatty acids. To look at the response of the taste cells to other fatty acids, I also tested several fatty acids that vary in their chain length and degree of unsaturation. Caproic acid (CA, C6:0) is a short chain saturated fatty acid; Myristic acid (MA, C14:0) is a medium chain saturated fatty acid; Oleic acid (OA, C18:1 cis-9) is a long chain mono-unsaturated fatty acid; Arachidonic acid (AA, C20:4 all-cis-5,8,11,14) is a long chain polyunsaturated fatty acid. All of the fatty acids tested initiated significant intracellular calcium changes in both type II and a subset of type III taste cells (Figure 3.5). The time courses of the intracellular calcium rise induced by different fatty acids varied. CA, MA, LA, and AA usually caused a delayed and gradual response, while OA initiated a much faster response. Besides the differences in the time course of their response profiles, the percentages of type II or III taste cells that responded to each of the fatty acids varied as well (Figure 3.6). Despite all these differences, it seems that the ability to activate a large subset of cells including both

type II and III taste cells is a common property shared by all types of fatty acids. At the same time, each taste cell can respond to several fatty acids (Figure 3.7), with some responding to all the fatty acids tested. This suggests the expression of a group of fatty acid-responsive proteins in these cells. Thus the coding mechanism for fat taste in the gustatory system seems to be very different from sweet, bitter and umami tastes where dedicated subsets of distinct type II taste cells encode the taste modalities and the selectivity for different taste qualities is determined by the nature of the receptors (see Chapter 1).

How does the taste system encode the signal of fat taste? This is still an open question. My studies showed that type II and III taste cells expressed different groups of fatty acid-responsive proteins and both responded to fatty acids. Other studies suggested that type I cells, which express GPR40, also respond to fatty acids (17). Thus it is possible that all three types of taste cells can respond to fat. Taste selectivity in the case of fat may be determined by the expression of different groups of fatty acid receptors and different downstream signaling pathways, e.g. type II taste cells that express fatty acid-activated GPCRs with CD36 may function as a “taste receptor cell”; type III taste cells that only express fatty acid-activated GPCRs may use fat to modulate the responses to other taste qualities. Alternatively, it is possible that all cells that express fatty acid-activated GPCRs are activated by fatty acids, and it is how they get activated and/or what neurotransmitters they release encodes the signal of fat taste.

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## CURRICULUM VITAE

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### EDUCATION

Ph.D. Candidate (Biology)                      Utah State University                      2005-2010  
Dissertation:    Fat Taste Transduction in Mouse Taste Cells: The Role of TRPM5

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### ACADEMIC PROJECTS

**Project # 1: Fat Taste Transduction** (Department of Biology, Utah State University)

The focus of this project is to study the mechanisms and processes for fat detection and how these pathways contribute to dietary selection and the control of food intake.

**Project # 2: Cell Typing of the Taste System** (Department of Biology, Utah State University)

The focus of this project is to study the different cell types in the taste system, utilizing normal and several transgenic animal models.

**Project # 3: Accessing genetic diversity of Chinese cultivated barley by STS markers** (Department of Biological Sciences, College of Life Sciences, Nankai University)

The focus of this project is to assess the genetic diversity among China's cultivated barley. To achieve this goal, sequence tagged site (STS) marker analysis was carried out to characterize 109 morphologically distinctive accessions originating from five Chinese eco-geographical zones. This project was evaluated top 3 of "100 Innovative Research Projects of Nankai University".



**Project # 4: Ecology Research of Tibet** (the Ministry of Science and Technology of China)

This well-known project was held by the Ministry of Science and Technology of China, providing a 3-month research opportunity for 28 outstanding undergraduate students selected from the whole country. I worked as the chairperson of this research group. The goal of this project is to study the natural vegetation and local animals of Tibet.

**TECHNICAL EXPERTISE**

- o **Patch clamp recording:** whole-cell and single channel recording; combined with rapid perfusion, pipette perfusion, flash photolysis
- o **Functional imaging:** calcium and other vital dye imaging in real time
- o **Molecular biology:** DNA/RNA isolation, RT-PCR, quantitative real-time PCR (qPCR), DNA/RNA analysis, sequencing, primer and probe design
- o **Immunocytochemistry and image analysis**
- o **In situ hybridization**
- o **Transepithelial current recording**
- o **Behavioral analysis:** preference testing, short-term lick monitoring, conditioned taste aversions, food intake analysis
- o **Statistical analysis:** SAS, R

**TEACHING EXPERIENCE**

**Course # 1: Neurobiology** (Department of Biology, Utah State University, 2009) My duty is to give some of the lectures, hold lab exercise on patch clamp recording and functional calcium imaging, organize discussions and reviews, and prepare exams, quizzes and assignments.

**Course # 2: Human Physiology** (Department of Biology, Utah State University, 2007-2008) My duty is to give some of the lectures, hold laboratory exercises, organize discussions and reviews, and prepare exams, quizzes and assignments.

**Course # 3: Biology Laboratory** (Department of Biology, Utah State University, 2005-2006) My duty is to hold laboratory exercises, organize discussions and reviews, and prepare exams, quizzes and assignments.

## AWARDS

- 2007 Val R. and Ruth Ann Christensen Student Leadership Scholarship, Utah State University
- 2008 AChemS Student Award, AChemS (Association for Chemoreception Sciences)
- 2009 Graduate Student Senate travel award, Utah State University
- 2010 The Graduate Student Professional Conference Award, Utah State University
- 2010 Graduate Student Travel & Research Grant, Utah State University
- 2010 VolkswagenStiftung Award, University of Hamburg & German Institute of Human Nutrition Potsdam-Rehbruecke

## INVITED PRESENTATIONS

1. Fatty acids induce increases in intracellular calcium in Type II and a subset of Type III mouse taste cells. (2009)  
Association for Chemoreception Sciences 31<sup>st</sup> Annual Meeting, Sarasota, FL.
2. A subpopulation of mouse Type II taste cells express functional voltage-gated calcium channels. (2010)  
Association for Chemoreception Sciences 32<sup>nd</sup> Annual Meeting, St. Pete Beach, FL.
3. Fatty acid transduction in mouse taste cells. (2010)  
30<sup>th</sup> Blankenese Conference, Hamburg-Blankenese, Germany.
4. Fatty acid transduction in mouse taste cells. (2010)  
Massachusetts General Hospital, Harvard Medical School.
5. Fatty acid transduction in mouse taste cells: how we taste fat? (2010)  
Neurobiology Department, Harvard Medical School.
6. Why is fat so tasty? (2010)  
Department of Biochemistry, Weill Cornell Medical College, Cornell University.

## PUBLICATIONS AND ABSTRACTS

1. Chen XW, Chen DF, **Liu P** (2006) Assessing Genetic Diversity of Chinese Cultivated Barley by STS Markers. *Genet Resour Crop Ev* 53:1665–1673.
2. Shah BP, **Liu P**, Yu T, Hansen DR, Gilbertson TA (2010) TRPM5 is critical for linoleic acid-induced CCK secretion from the enteroendocrine cell line, STC-1. (submitted to *J Biol Chem*)

3. Shah BP, Yu T, **Liu P**, Gilbertson TA (2010) Direct evidence of the role of TRPM5 in bitter transduction in the enteroendocrine cell line, STC-1. (submitted to *J Biochem*).
4. **Liu P**, Shah BP, Croasdell S, Gilbertson TA (2010) TRPM5 is essential for fat taste. (submitted to *J Neurosci*).
5. **Liu P**, Gilbertson TA (2010) Fatty acids depolarize and elicit a rise in intracellular Ca<sup>2+</sup> in Type II and a subset of Type III mouse taste cells. (submitted to *J Neurosci*).
6. Yu T, Shah BP, **Liu P**, Hansen DR, Gilbertson TA (2007) Fatty acid-induced changes in intracellular calcium in gustatory and somatosensory cells: Mechanisms underlying the taste and texture of fat. Society for Neuroscience Annual Meeting, San Diego, CA.
7. Shah B, **Liu P**, Yu T, Hansen DR, Gilbertson TA (2008) RNA interference of GPR120 inhibits responses to fatty acids in the enteroendocrine cell line, STC-1: Implications for fatty acid transduction. International Symposium for Olfaction & Taste and Association for Chemoreception Sciences 30<sup>th</sup> Annual Meeting, San Francisco, CA.
8. Yu T, Shah B, **Liu P**, Hansen DR, Gilbertson TA (2008) Fatty acid-induced changes in intracellular calcium in somatosensory cells: Mechanisms underlying the textural perception of fat. International Symposium for Olfaction & Taste and Association for Chemoreception Sciences 30<sup>th</sup> Annual Meeting, San Francisco, CA.
9. **Liu P**, Yu T, Shah B, Hansen DR, Gilbertson TA (2008) Fatty acids elicit membrane and a rise in intracellular calcium in rodent taste cells. International Symposium for Olfaction & Taste and Association for Chemoreception Sciences 30<sup>th</sup> Annual Meeting, San Francisco, CA.
10. **Liu P**, Gilbertson TA (2009) Fatty Acids Elicit A Robust Rise in Intracellular Calcium in Mouse Taste Bud Cells. Chemical Senses: Receptors and Circuits, Keystone Symposia, Tahoe City, CA.
11. **Liu P**, Shah BP, Hadawar H, Gilbertson TA (2009) Fatty acids induce increases in intracellular calcium in Type II and a subset of Type III mouse taste cells. Association for Chemoreception Sciences 31<sup>st</sup> Annual Meeting, Sarasota, FL.
12. Shah BP, **Liu P**, Yu T, Hansen DR, Gilbertson TA (2009) Direct evidence of the role of TRPM5 in bitter transduction in enteroendocrine cells. Association for Chemoreception Sciences 31<sup>st</sup> Annual Meeting, Sarasota, FL.
13. Yu T, Shah BP, **Liu P**, Gilbertson TA (2009) Fatty acid transduction in chemosensory cells. Association for Chemoreception Sciences 31<sup>st</sup> Annual Meeting, Sarasota, FL.

14. **Liu P**, Gilbertson TA (2010) A subpopulation of mouse Type II taste cells express functional voltage-gated calcium channels. Association for Chemoreception Sciences 32<sup>nd</sup> Annual Meeting, St. Pete Beach, FL.
15. Gilbertson TA, **Liu P**, Margolskee RF (2010) TRPM5 is required for fatty acid transduction in mouse taste cells. Association for Chemoreception Sciences 32<sup>nd</sup> Annual Meeting, St. Pete Beach, FL.
16. **Liu P**, Gilbertson TA (2010) Fatty acid transduction in mouse taste cells. 30<sup>th</sup> Blankenese Conference, Hamburg-Blankenese, Germany.

## **MEMBERSHIP**

- o Member of Association for Chemoreception Sciences, USA 2006-2010
- o Vice President of CSSA (Chinese Students and Scholars Association), USU 2005-2006